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Effect of Respiratory Syncytial Virus on Apnea in Weanling Rats

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ABSTRACT

Apnea is a common complication of respiratory syncytial virus (RSV) infection in young infants. The purpose of this study was to determine whether this infection affects apnea triggered by sensorineural stimulation in weanling rats. We also studied which neurotransmitters are involved in this response and whether passive prophylaxis with a specific neutralizing antibody (palivizumab) confers protection against it. Weanling rats were inoculated intranasally with RSV, adenovirus, or virus-free medium. Changes in respiratory rate and apnea in response to nerve stimulation with increasing doses of capsaicin were measured by plethysmography. Capsaicin-induced apnea was significantly longer in RSV-infected rats at postinoculation days 2 (upper airways infection) and 5 (lower airways infection), and apnea-related mortality occurred only in the RSV-infected group. Reduction in the duration of apnea was observed after selective inhibition of central γ-aminobutyric acid (GABA) type A receptors and neurokinin type 1 receptors for substance P. Prophylactic palivizumab protected against apnea and apnea-related mortality. These results suggest that sensorineural stimulation during RSV infection is associated with the development of apnea and apnea-related death in early life, whose mechanism involves the release of GABA acting on central GABA type A receptors and substance P acting on neurokinin type 1 receptors. **(*Pediatr Res* 57: 819–825, 2005)**

Abbreviations

CGRP, calcitonin gene-related peptide
GABA, γ-aminobutyric acid
$GABA_A$, γ-aminobutyric acid receptor type A
NK_1, neurokinin 1
RSV, respiratory syncytial virus
SID, sudden infant death
TRPV, transient receptor potential channel, vanilloid subfamily

Respiratory syncytial virus (RSV) is the most important cause of lower respiratory tract infections in infants and young children and presents a large public health problem worldwide (1). Although most infections have a mild course, RSV can cause severe respiratory compromise, especially in patients with underlying cardiorespiratory conditions, and predisposes to the development of childhood asthma (2). Apnea can be the presenting sign of this infection, and its incidence varies between 16 and 25% of infected infants, with a particularly high risk associated with young age (<3 mo) and prematurity (3–5). Because of its ability to interfere with ventilatory control, it has been suggested that RSV infection may also be a precipitating factor in cases of sudden infant death (SID) (6–8), yet the pathophysiology of apnea associated with RSV infection remains unclear, and no specific prophylaxis or treatment is currently available to protect infants from this complication.

Among the factors that influence airway function are neural control mechanisms, which can undergo dysregulation during and after viral respiratory infections (9). In particular, the local irritation of C-type nociceptive afferents in infected airways evokes bronchospasm, neurogenic inflammatory edema, and recruitment and activation of leukocytes (10). We showed previously that RSV infection amplifies the inflammatory and immunomodulatory effects of sensory nerves *via* both presynaptic (increased synthesis and release of substance P) and postsynaptic [up-regulation of neurokinin 1 (NK_1) receptors] mechanisms (11–13), both involving the overexpression of nerve growth factor by the infected respiratory epithelium (14).

Sensorineural stimulation releases multiple neurotransmitters that affect the control of ventilation and can trigger apnea

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(15). Thus, the primary purpose of this study was to determine whether RSV infection of the upper or lower respiratory tract affects the development and duration of apnea in rats that were stimulated with capsaicin, a neurotoxin that binds the transient receptor potential channel vanilloid subfamily member 1 ($TRPV_1$) and is highly expressed on C-type afferent neurons (16). We repeated some of these experiments using a different viral pathogen, adenovirus, to verify whether the effect of RSV was specific or rather a nonspecific consequence of virus-induced airway inflammation. Furthermore, we explored the mechanism of apnea in our model by blocking different neurotransmitters [substance P, calcitonin gene-related peptide (CGRP), and γ-aminobutyric acid (GABA)] that are released directly or indirectly by capsaicin and known to affect the control of ventilation (15,17–19). Finally, we studied whether the administration of RSV-neutralizing antibodies protects against capsaicin-induced apnea and apnea-related mortality in RSV-infected rats. To this end, we investigated a humanized MAb against the RSV fusion (F) protein (20) used widely for the prophylaxis of this infection in high-risk children (21).

METHODS

Animals. We used weanling pathogen-free F-344 rats that were born in our laboratory from dams that were obtained from Taconic Laboratories (Germantown, NY) and maintained under strict barrier conditions. Average body weight of the weanlings at the time of the experiment was 37 ± 4 g. All animals used in this study were maintained under strict barrier conditions from birth until they were killed to prevent any microbial contamination. Each dam or up to two litters were housed in polycarbonate cages isolated by polyester filter covers. All cages were placed on racks that provided positive individual ventilation with class 100 air to each cage at the rate of approximately one cage change of air per minute (Maxi-Miser; Thoren Caging Systems, Hazleton, PA). We used separate rooms for housing infected and pathogen-free weanling rats, both serviced by specially trained husbandry technicians. All manipulations were conducted inside a class 100 laminar flow hood. Bedding, water, and feed were autoclaved before use, and the packages were opened only under the laminar flow hood. Cages and water bottles were passed through a tunnel washer after each use and disinfected with both chemical and heat. The experimental procedures followed in this study were approved by the Animal Care and Use Committee of the University of Miami School of Medicine.

Virus preparation and inoculation. HEp-2 cells were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (GIBCO-BRL, Grand Island, NY). Confluent monolayers of HEp-2 cells were infected with 0.1 plaque forming units of human RSV strain A_{Long}, and the infection was allowed to proceed at 37°C in 5% CO_2 atmosphere until >75% of the cells exhibited cytopathic effect. Cell debris was removed by centrifugation at 9500 × *g* for 20 min in a refrigerated centrifuge (4°C). Aliquots of the virus stock were snap-frozen in liquid nitrogen and stored at −70°C. Before inoculation, the viral stock was titrated and diluted as needed for a final titer of 3.5×10^7 pfu/mL. Supernatants and cell lysates from virus-free flasks of HEp-2 cells in minimum essential medium were harvested, centrifuged, and aliquotted following the same protocol to obtain the virus-free medium that was used as a sham infection control.

Weanling rats at 2 wk of age were inoculated intranasally under sodium pentobarbital anesthesia (50 mg/kg i.p.) by depositing in each nostril 20 μL of virus suspension that contained 3.5×10^7 pfu/mL (12). For the studies with adenovirus, we inoculated the same volume and same titer of human adenovirus type 4 obtained from American Type Culture Collection (Manassas, VA) (22).

RSV titration by real-time PCR. Total RNA was extracted from whole-lung homogenates in 1 mL/100 mg tissue of RNA STAT-60 solution (Tel-Test, Friendswood, TX) and subsequently treated with the DNase-free kit (Ambion, Austin, TX). A qualitative agarose gel was run for all DNase I–treated samples to confirm RNA integrity before amplification. cDNA synthesis was accomplished using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). Briefly, 1.0 μg of DNase I–treated total RNA was used in a 20-μL final reaction volume that contained 20 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM of each dNTP, 0.1 μg of random hexamers, and 50 U of SuperScript III RTase. Samples were incubated at 42°C for 50 min followed by a termination step at 85°C for 5 min. A final RNase H step was performed by adding 1 μL of enzyme to each sample and incubating at 37°C for 20 min. For generating a standard curve, viral RNA was isolated from 10-fold serial dilutions of our RSV stock using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA), and cDNA was generated as described above using 1/10 volume of isolation eluates (~6 μL). The RSV PCR product was cloned into the pCR 2.1-TOPO vector using the TOPO TA Cloning Kit (Invitrogen), and sequence was verified. Real-time PCR reactions were performed using a LightCycler instrument and the FastStart DNA Master Hybridization Probes kit (Roche Applied Science, Indianapolis, IN). Briefly, 2-μL aliquots of the synthesized cDNA were added to an 18-μL PCR mixture that contained 4 mM $MgCl_2$, 0.5 μM of each primer, 0.2 μM of each probe, and 1× FastStart Reaction Mix. RSV primers and probes were designed from GenBank accession number M11486: 5'-GCGATGTCTAGGTTAGGA-3' (sense), 5'-GCTATGTCCTTGGGTAGT-3' (antisense), 5'-AGGTAGCTCCAGAATACAGGC-fluorescein-3' (5' probe), 5'-LC Red640-GACTCTCCTGATTGTGGGATGA-phosphate-3' (3' probe). Cycling conditions consisted of an initial 10-min denaturation at 95°C, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 20 s. Quantification was performed by extrapolating copy numbers from a standard curve using the Second Derivative Maximum Method with the LightCycler software.

A sample of RSV stock was subjected to 12 10-fold serial dilutions, and viral RNA was subsequently isolated from each dilution. Adjusting for volume changes at each step (including cDNA synthesis), we prepared concentration standards spanning 5×10^4 to 5×10^{-7} pfu/mL. A dynamic range of linear relationship was observed over 8 orders of magnitude, demonstrating sensitivity down to 5×10^{-3} pfu/mL. Concentrations ranging from 5×10^4 to 5×10^{-3} pfu/mL were plotted against cycle number to generate a linear regression standard curve (Fig. 1). Upper airway (nasal mucosa) and lower airway tissues were harvested at the time of killing, snap-frozen in liquid nitrogen, and processed separately for total RNA isolation and virus titration.

Central venous catheter placement. One day before the experiment, a catheter (Silastic tubing, 0.020 × 0.37 in; Dow Corning, Midland, MI) was placed in the right external jugular vein under sodium pentobarbital anesthesia and was advanced until its tip was slightly above the right atrium. The exact length necessary to position the catheter was measured previously in weanling rats of comparable size. Once placed, the catheter was drawn s.c., exteriorized dorsally between the scapulae, and filled with heparin (100 units/mL).

Ventilatory measurements. Two or 5 d after inoculation, unanesthetized weanling rats were placed in a body plethysmograph for the measurements of ventilatory parameters (Buxco Co., Sharon, CT). Rats were acclimated to the chamber for 60 min before administration of any drug challenge. The rat was able to move inside the plethysmograph but remained attached to a swivel that contained the vascular catheter, which ran from the connector sutured in its skin to the top portion of the chamber. The respiratory signal measured by a transducer was recorded on a polygraph for subsequent analysis. Capsaicin was diluted in 0.9% NaCl and was administered at increasing doses (1, 2.5, 5, and 10 μg/kg i.v.) *via* the central venous catheter with a 10-min interval between doses.

Neurotransmitter blockers. Capsaicin affects directly or indirectly the release of multiple neurotransmitters that are involved in the control of breathing at either a central or a peripheral level. These include substance P (15), CGRP (18), and GABA (23). The following pharmacologic blockers

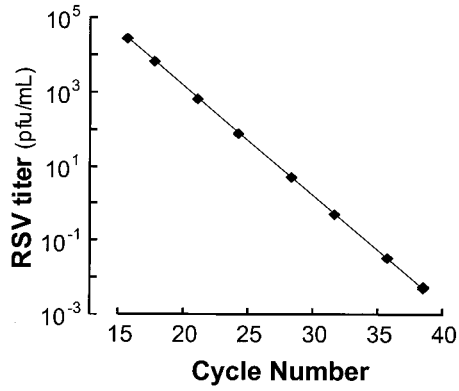

Figure 1. Real-time PCR titration of RSV. A dynamic range of linear relationship was observed over 8 orders of magnitude, demonstrating sensitivity down to 5×10^{-3} pfu/mL. Concentrations ranging from 5×10^4 to 5×10^{-3} pfu/mL were plotted against cycle number to generate a linear regression standard curve.

were used to assess the role played by each transmitter in our model: *1*) CP-122721, inhibitor of the high-affinity substance P receptor (NK_1), 10 mg/kg s.c. 1 h before capsaicin (24); *2*) $CGRP_{8-37}$, inhibitor of the $CGRP_1$ receptor, 150 μg/kg i.v. 10 min before capsaicin (18); *3*) bicuculline, inhibitor of the $GABA_A$ receptor able to cross the blood-brain barrier, 0.3 mg/kg i.v. 10 min before capsaicin (23,25,26); and *4*) bicuculline methiodide, inhibitor of peripheral $GABA_A$ receptors unable to cross the blood-brain barrier, 3 mg/kg i.v. 10 min before capsaicin (26). Controls received an injection of 0.9% NaCl (1 mL/kg i.v.), which was the vehicle used as the diluent for all inhibitors.

RSV-neutralizing antibody. For testing the effect of passive prophylaxis against RSV, infected rats were treated with an injection of either palivizumab (MedImmune, Inc., Gaithersburg, MD; 15 mg/kg i.p.) or its vehicle (0.9% NaCl, 1 mL/kg i.p.). The dose of palivizumab used in this study corresponds to the dose currently used in routine clinical practice (21) on the basis of previous studies conducted in animal models (20,27).

Experimental protocols. In preliminary experiments, we localized the RSV infection to the upper or lower airways by real-time PCR analysis of the nasal mucosa and trachea at different time points after inoculation ($n = 3$ rats per time point). We also analyzed the ventilatory pattern of weanling rats that were inoculated with RSV or virus-free medium in the absence of nerve stimulation ($n = 5$ rats per group).

For determining whether RSV infection affects capsaicin-induced apnea, weanling rats ($n = 6$–9 rats per group) were inoculated intranasally with RSV or virus-free medium and placed unanesthetized in a plethysmograph to assess their responses to increasing doses of capsaicin. For exploring the time course of these responses, the capsaicin challenge was performed at 2 or 5 d after inoculation (*i.e.* at 16 or 19 d of age). We chose these time points to discriminate the impact of upper and lower respiratory infection, respectively, on the basis of our real-time PCR experiments.

To determine whether the effect of RSV on capsaicin-induced apnea was specific or rather the nonspecific consequence of virus-induced airway inflammation, we tested the effect of adenovirus *versus* virus-free medium at 2 d after inoculation ($n = 6$ per group). Adenovirus was chosen because it is a common respiratory pathogen, potentiates airway neurogenic inflammation in our model in a manner similar to RSV (22), and has not been associated with the development of apnea in infants (3).

To study the role of individual neurotransmitters in our model, we administered selective receptor blockers to rats that were infected 2 d earlier with RSV before the challenge with capsaicin ($n = 6$ rats per each blocker). In each experiment with pharmacologic blockers, controls were pathogen-free and RSV-infected weanling rats that received an injection of vehicle before the challenge with capsaicin ($n = 6$ rats per group).

For studying the protective effect of passive prophylaxis, groups of pathogen-free weanling rats were treated with palivizumab 24 h before RSV inoculation, and capsaicin-induced apnea was measured at 2 or 5 d after inoculation ($n = 6$ rats per group). For studying the effect of palivizumab given during an established infection, the antibody was injected 72 h after RSV inoculation and capsaicin-induced apnea was measured 5 d after inoculation. In each experiment, controls were rats that were inoculated with virus-free medium or RSV and received an injection of the vehicle of palivizumab at the same time points ($n = 6$–9 rats per group). We also studied apnea-related mortality after capsaicin with or without palivizumab protection ($n = 6$–9 rats per group).

Statistical analysis. Continuous data are expressed as the mean ± SEM. Mean values of apnea duration in seconds were analyzed by repeated measures ANOVA (28). Mortality was expressed as proportions and analyzed by χ^2 test. Multiple comparisons between means were performed with Fisher protected least significant difference test (29). Statistical analysis was performed using the StatView software version 5.0.1 (SAS Institute, Cary, NC). Differences at $p < 0.05$ were considered significant.

RESULTS

Localization of RSV infection. Two days after inoculation, high RSV titers were detected by real-time PCR analysis in the upper airways of all rats that were inoculated with the virus ($6.8 \pm 4.4 \times 10^{-2}$ pfu/mL), whereas no detectable virus was found in the lower airways of the same rats. In contrast, RSV was readily detectable in the lower airways 5 d after inoculation ($1.6 \pm 1.1 \times 10^{-2}$ pfu/mL). Therefore, we adopted the 2-d time point as a model of infection limited to the upper airways and the 5-d time point as a model of infection spreading through the lower airways.

Effect of RSV infection. In preliminary experiments, we analyzed the respiratory rate of weanling rats that were inoculated with RSV or virus-free medium in the absence of nerve stimulation ($n = 5$ rats per group) and found that RSV infection *per se* did not have any direct effect. The respiratory rate recorded in rats that were inoculated with virus-free medium (168 ± 5 breaths/min) was similar to that measured in rats that were inoculated with RSV (172 ± 5; $p = 0.5$); furthermore, we were not able to detect apnea or hypopnea in any unstimulated pathogen-free or RSV-infected rat. Also, infusion of the vehicle of capsaicin had no effect on the respiratory rate of pathogen-free (174 ± 5; $p = 0.4$) or RSV-infected rats (173 ± 5; $p = 0.9$) and did not induce any episode of apnea.

Effect of capsaicin. No apnea was detected after the injection of 1 μg/kg capsaicin either 2 or 5 d after inoculation of virus-free medium or RSV ($n = 6$–9 rats per group). However, weanling rats consistently developed apnea in a dose-dependent manner when challenged with capsaicin at doses >1 μg/kg, and the duration of these episodes of apnea was longer in rats that were inoculated with RSV compared with controls that were dosed with virus-free medium. Two days after inoculation (Fig. 2*A*), apnea was significantly longer in RSV-infected rats that were challenged with 5 μg/kg capsaicin compared with pathogen-free controls (mean difference = 6.8 s; $p = 0.026$), and an even greater difference was observed after the 10-μg/kg dose (25.9 s; $p = 0.0001$). The difference measured at the 2.5-μg/kg dose was not statistically significant (3.6 s; $p = 0.19$).

Five days after inoculation (Fig. 2*B*), the apnea triggered by capsaicin was potentiated in infected rats at all capsaicin doses studied. Again, the difference between RSV-infected and pathogen-free rats was maximal after 10 μg/kg of capsaicin (24.8 s; $p = 0.0002$). When we compared the effect of capsaicin in infected rats at 5 *versus* 2 d after inoculation (Fig. 2*C*), we found that the duration of apnea at the 2.5-μg/kg dose was increased significantly at 5 d after infection ($p = 0.043$), whereas the differences found between the two time points after 5 μg/kg ($p = 0.19$) and 10 μg/kg ($p = 0.78$) of capsaicin were not significant. On the basis of these data, we elected to analyze the effects of neurotransmitter blockers and RSV-neutralizing antibodies described below using capsaicin at the dose of 10 μg/kg given 2 d after inoculation. When we repeated the same experimental protocol comparing rats that were inoculated with adenovirus with controls that were dosed with virus-free medium at 2 d after inoculation (Fig. 3), we found no significant difference after the injection of capsaicin at the doses of 2.5 μg/kg (−1.5 s; $p = 0.7$), 5 μg/kg (−2.5 s; $p = 0.3$), and 10 μg/kg (1.0 s; $p = 0.7$).

Effects of neurotransmitter blockers. We analyzed the effect of blocking selected neurotransmitters that are involved in control of breathing ($n = 6$ rats per each blocker) on the apnea response triggered by 10 μg/kg capsaicin in weanling rats that were inoculated 2 d earlier with RSV (Fig. 4). Selective antagonism of the $GABA_A$ receptor with bicuculline abolished the potentiating effect of RSV infection on capsaicin-induced apnea ($p = 0.0001$). In contrast, this inhibitory effect was not present when the infected rats were pretreated with bicuculline methiodide, a $GABA_A$ receptor antagonist that acts only peripherally without crossing the blood-brain barrier ($p = 0.54$).

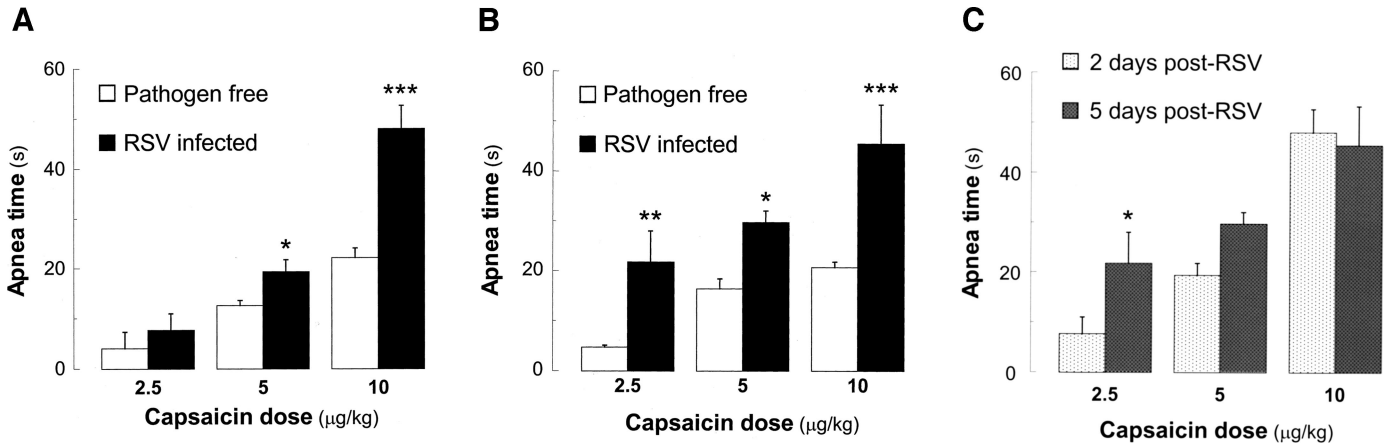

Figure 2. Effect of RSV upper respiratory infection on capsaicin-induced apnea in weanling rats. Rats were inoculated with virus-free medium or RSV suspension 2 d (*A*) or 5 d (*B*) before stimulation with increasing doses of capsaicin. Capsaicin-induced apnea was significantly longer in infected rats at both time points. The response to the lowest dose of capsaicin was significantly longer at the later time point, whereas no difference was found at higher levels of stimulation (*C*). Bars show mean ± SEM; $n = 6$–9 rats per group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ = significantly different from pathogen-free controls.

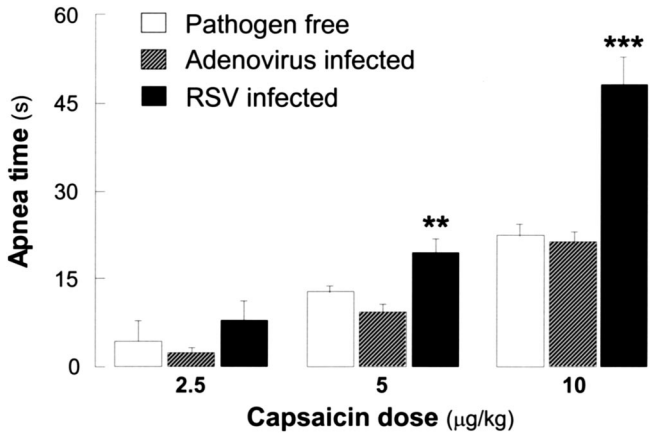

Figure 3. Comparison of the effects of RSV *vs* adenovirus lower respiratory infection on capsaicin-induced apnea in weanling rats. Rats were inoculated with virus-free medium, adenovirus suspension, or RSV suspension 5 d before stimulation with increasing doses of capsaicin. Apnea duration was significantly longer in RSV-infected rats, whereas adenovirus infection did not have any significant effect. Bars show mean ± SEM; $n = 6$ rats per group. **$p < 0.01$; ***$p < 0.001$ = significantly different from pathogen-free controls.

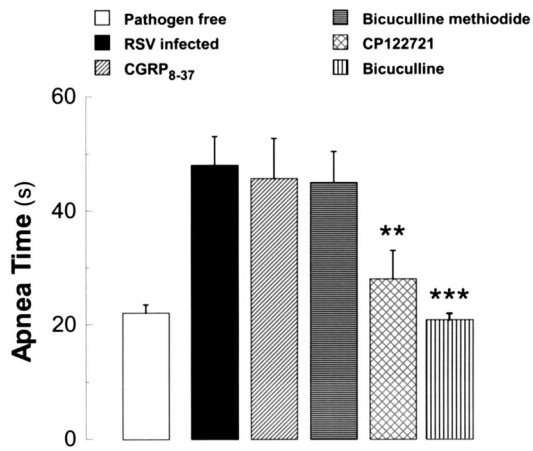

Figure 4. Effect of selective neurotransmitter receptor blockers against capsaicin-induced apnea in RSV-infected weanling rats. Rats were inoculated with virus-free medium or RSV suspension 2 d before stimulation with 10 μg/kg capsaicin. Capsaicin-induced apnea was prevented by selective blockade of NK_1-type substance P receptors with CP-122721 or central $GABA_A$ receptors with bicuculline. Blockade of $CGRP_1$ receptors with $CGRP_{8-37}$ or peripheral $GABA_A$ receptors with bicuculline methiodide had no effect. Bars show mean ± SEM; $n = 6$ rats per group. **$p < 0.01$; ***$p < 0.001$ = significantly different from RSV-infected, capsaicin-stimulated rats that received an injection of the vehicle used as the diluent for the inhibitors.

Pretreatment of RSV-infected rats with the selective NK_1 receptor antagonist CP-122721 also decreased the duration of apnea evoked by sensorineural stimulation ($p = 0.008$). After pretreatment with CP-122721 or bicuculline, the apnea response to capsaicin in RSV-infected weanling rats was similar to pathogen-free controls ($p = 0.33$ and 0.76, respectively). $CGRP_{8-37}$ did not affect the apnea response to capsaicin in RSV-infected rats ($p = 0.7$).

Effect of RSV-neutralizing antibody. Passive prophylaxis given 24 h before the inoculation of RSV (Fig. 5) prevented apnea triggered by stimulation with 10 μg/kg capsaicin ($n = 6$–9 rats per group) at 2 d ($p = 0.0002$) and 5 d after inoculation ($p = 0.0001$). There was no significant difference between the infected rats that received prophylaxis and pathogen-free controls at 2 d ($p = 0.1$) or 5 d ($p = 0.46$) after inoculation. Palivizumab administered 72 h after RSV inoculation reduced apnea significantly in infected rats ($p = 0.029$) at 5 d after inoculation but was not as effective as when given before the infection.

No mortality was observed as a result of RSV infection alone or capsaicin stimulation alone ($n = 6$–9 rats per group). However, the combination of RSV infection and sensorineural stimulation with capsaicin was associated with significant mortality (Fig. 6): 2 d after RSV inoculation, mortality after capsaicin stimulation was 66%, whereas none of the pathogen-free controls died after capsaicin ($p = 0.01$). Also, no apnea-related mortality was seen in the adenovirus-infected rats that were stimulated with the same doses of capsaicin 2 d after inoculation. Mortality among RSV-infected, capsaicin-stimulated rats decreased to 22% at 5 d after infection and was no longer statistically different from controls ($p = 0.3$).

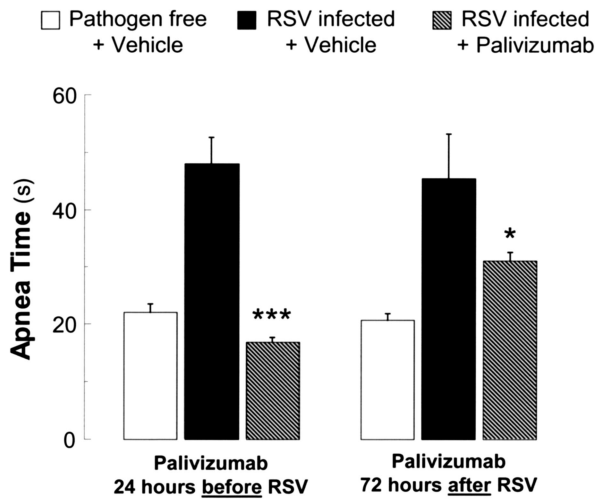

Figure 5. Effect of RSV-neutralizing antibodies against capsaicin-induced apnea in RSV-infected weanling rats. Rats were inoculated with virus-free medium or RSV suspension 2 d before stimulation with 10 μg/kg capsaicin. A humanized MAb against the RSV F protein (palivizumab) was injected 24 h before or 72 h after inoculation. RSV-induced potentiation of capsaicin-induced apnea was prevented when the antibody was given before the infection and was significantly inhibited when the antibody was given during the infection. Bars show mean ± SEM; $n = 6$–9 rats per group. *$p < 0.05$; ***$p < 0.001$ = significantly different from RSV-infected, capsaicin-stimulated rats that received an injection of the vehicle of palivizumab.

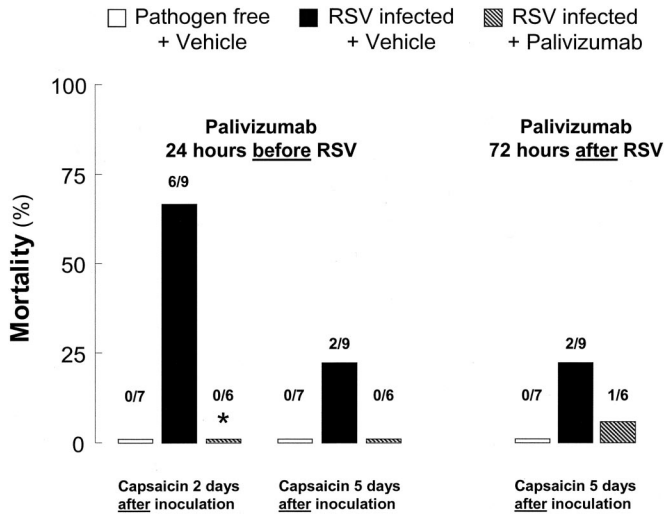

Figure 6. Effect of RSV-neutralizing antibodies against apnea-related mortality in RSV-infected weanling rats. Rats were inoculated with virus-free medium or RSV suspension 2 or 5 d before stimulation with 10 μg/kg capsaicin. Palivizumab was injected 24 h before or 72 h after inoculation. Apnea-related mortality was higher 2 d after inoculation and was abolished by passive prophylaxis. Bars show mean ± SEM; $n = 6$–9 rats per group. *$p < 0.05$ = significantly different from RSV-infected rats that received an injection of the vehicle of palivizumab.

Passive prophylaxis protected RSV-infected rats against apnea-related mortality after capsaicin stimulation at 2 d after infection ($p = 0.017$). No mortality was also observed in the prophylaxis group that was stimulated at 5 d after infection, but this effect was not statistically significant because of the lower mortality in the RSV control group without prophylaxis ($p = 0.34$). Similarly, the effect of palivizumab administered 72 h after infection was not statistically significant ($p = 0.66$).

DISCUSSION

This study shows for the first time that early-life RSV-infection in rats is associated with significant prolongation of the apnea triggered by sensorineural stimulation. This effect is already significant 2 d after inoculation of the virus, *i.e.* during the upper respiratory phase of the infection, and is still present after 5 d of infection, *i.e.* after spreading of the infection to the lower respiratory tract. The main difference between the two phases of the infectious process is that mortality is much higher early in the course of the infection, suggesting that protective mechanisms (*e.g.* autoresuscitation) may not be activated or mature fully until a later time point. Also, the response to low-level stimulation becomes significant only at the later time point. In other words, with the spreading of the infection to the lower respiratory tract, rats have a lower threshold for apnea but are also less likely to die from it. The effect of RSV in our model is age specific as preliminary studies with adult F-344 rats did not show any mortality associated with capsaicin-induced apnea during the infection (30), which is consistent with the notion that this complication is typical of the first months of life (4,5).

The susceptibility to apnea and apnea-related mortality during the early phase of the infection suggests that RSV can produce serious disruption of the neural control of breathing even when the infection is still confined to the upper airways, which provides a rationale to the clinical observation that apnea is frequently the presenting symptom of the infection and typically precedes the appearance of coryza, cough, or wheezing (3,4). Furthermore, our findings provide a pathophysiologic basis to several parallels that have been drawn between RSV infections and SID, such as the high incidence in winter months, the occurrence in young infants 2–4 mo of age, the increased risk for premature-born infants, and the frequent report of upper respiratory tract symptoms in the days preceding SID (31). This hypothesis is also supported by the frequent histopathologic detection of RSV on postmortem examination in the lungs of infants who die of SID (32).

Apnea related to respiratory infections in infants is also seen with other viruses (3,33), but the proportion of patients who develop this complication is much smaller than with RSV. A retrospective study of hospitalized infants who were <6 mo of age found that the combined incidence of apnea associated with respiratory viruses other than RSV (including influenza, parainfluenza type 3, and rhinovirus) was 8.8% compared with 20.4% in the RSV group (3). In the same study, of 57 infants who were infected with adenovirus, none developed apnea. Adenovirus has been tested previously in our F-344 rat model and found to potentiate neurogenic inflammation much like RSV (22); therefore, we elected to use this virus as a control to find whether the potentiation of capsaicin-induced apnea is a specific effect of RSV or rather a nonspecific consequence of airway neurogenic inflammation. Capsaicin-induced apnea in adenovirus-infected weanling rats was not different from pathogen-free controls, and no mortality was noted in either group, suggesting that RSV has a unique influence on control of breathing in early life.

Mechanisms. The exact mechanisms whereby RSV infection favors the development of apnea are still unclear, although

it is generally accepted that RSV-associated apnea is caused by central inhibition of the ventilatory drive, with complete absence of respiratory effort and without evidence of airway obstruction (4). As a trigger for apnea in our study, we used capsaicin, a neurotoxin that binds a calcium permeable channel ($TRPV_1$) that is expressed predominantly by C-type unmyelinated sensory neurons (34). This receptor responds to a variety of microenvironmental stimuli (chemical irritants, heat, acid pH) by co-releasing multiple neurotransmitters, including substance P, neurokinins, CGRP, vasoactive intestinal peptide, and somatostatin. Because multiple stimuli can converge on the same TRPV channel, these channels also function as a site of integration of diverse physical and chemical signals toward the same transduction pathway (35).

Capsaicin-induced apnea depends on stimulation of primary neurons within the nodose ganglia, with the afferent path from the airway mucosa traveling *via* the superior laryngeal nerves (36). This inhibitory pathway has been implicated in the pathophysiology of apnea (37) and SID (38) in infants and is known to operate *via* centrally mediated GABAergic pathways (23) that activate $GABA_A$ ionotropic fast chloride receptors. This mechanism undergoes developmental maturation, as shown by the observation that the $GABA_A$ receptor inhibitor bicuculline blocks the laryngeal inhibitory reflex almost exclusively in young animals and preterm infants (39).

Our present study shows that the mechanism of capsaicin-induced apnea during RSV infection involves the activation of $GABA_A$ receptors within the CNS, but it is also modulated by substance P *via* its high-affinity NK_1 receptors. We reported previously that RSV infection in rats produces a strong potentiation of neurogenic-mediated inflammation by amplifying the synthesis and release of substance P (13) and the expression of NK_1 receptors on target cells (11,12), thus making the airways abnormally susceptible to the proinflammatory effects of this peptide primarily released from sensory nerves (11). This complex remodeling of sensorineural pathways in the respiratory tract is coordinated *via* increased expression of the prototypical neurotrophin nerve growth factor from infected epithelia (14). Although capsaicin does not release GABA directly (40), it is known that substance P released from primary afferents activates (41) and up-regulates (42) second-order GABAergic interneurons in the spinal dorsal horn, an inhibitory mechanism that modulates the spinal transmission of nociceptive information. Centrally, these GABAergic pathways project to medullary inspiratory neurons, hyperpolarize their membrane by increasing permeability to chloride ions, and inhibit their function resulting in apnea.

Therefore, we speculate that the previously reported pre- and postsynaptic remodeling induced by RSV in primary nociceptive afferents (10–13) may result in overstimulation of inhibitory GABAergic pathways in infants and in turn enhance their vulnerability to develop apnea whenever irritative stimuli are applied to the respiratory mucosa. These stimuli are likely to include any agent that is able to activate TRPV channels, such as the extracellular acidification resulting from gastroesophageal reflux, overheating caused by microclimatic conditions or sleeping in prone position, and the exposure to indoor pollutants such as environmental tobacco smoke or other fine particles.

According to this model, the infection alone is necessary but not sufficient to cause apnea or SID; rather, a combination of events or "double hit" is required. The first hit derives from the RSV infection and the consequent resetting of sensorineural and GABAergic pathways, whereas the second hit derives from one or more intrinsic (*e.g.* gastroesophageal reflux) or extrinsic (*e.g.* environmental tobacco smoke exposure) events triggering the TRPV channels expressed on airway sensory nerves. The requirement of a second hit can also explain why only one of four or five infants who are infected with RSV develops apnea. Of course, additional animal studies and eventually human studies are necessary to substantiate this hypothesis.

Prevention. This study shows for the first time that the aberrant apnea response to sensorineural stimulation and the associated mortality observed during RSV infection can be prevented if passive prophylaxis with RSV-neutralizing antibodies is administered before the onset of the infection. Even more important, RSV-neutralizing antibodies retain a strong protective activity against the development of apnea when given in the early stage of the infection. The latter observation may have important clinical implications because apnea is frequently the presenting sign of the infection with RSV; therefore, it is conceivable that the prompt administration in young infants with an unexplained apneic episode may be beneficial. However, because mortality is highest during the first 48 h of the infection, our data suggest that prophylaxis should be preferred for the protection of high-risk infants (*e.g.* young premature infants), especially against the increased risk for SID (43). Again, the protective effect of RSV-neutralizing antibodies found in our animal model needs confirmation from specifically designed clinical studies.

Palivizumab is a humanized IgG-1 MAb against the surface F glycoprotein of the virus, which mediates viral penetration into the airway epithelial cells (20). This antibody is currently indicated for use in high-risk infants as a monthly intramuscular injection during the RSV season to prevent serious infections (21). A newer generation antibody has been developed recently (Numax; MedImmune, Inc., Gaithersburg, MD), which binds 50-fold better to the RSV F protein, is 20-fold more potent *in vitro* at neutralizing RSV, and is 10-fold more potent *in vivo* in cotton rats than palivizumab (44) and therefore may offer better protection against apnea and apnea-related mortality, especially when used during established infections.

CONCLUSION

In this study, we report for the first time that RSV infection in rats prolongs significantly the apnea triggered by sensorineural stimulation. Also, stimulation during the infection is followed by apnea-related mortality in up to two thirds of the rats, whereas the same level of stimulation is never lethal in noninfected rats or in rats that are infected with adenovirus. The effect of RSV on ventilatory control is already significant 2 d after intranasal inoculation, when the infection is still confined to the upper airway. This finding is consistent with and explains previous epidemiologic reports indicating that apnea is an early or even presenting manifestation of this infection. In addition, apnea-related mortality

is highest early during the infection, suggesting that counterregulatory mechanisms that facilitate autoresuscitation are activated during the infection.

Specific blockade of the central $GABA_A$ receptors or of the high-affinity substance P (NK_1) receptors abolishes the influence of RSV infection on the apnea triggered by sensorineural stimulation. This finding suggests that substance P released from primary sensory neurons within the nodose ganglia activates second-order GABAergic interneurons in the spinal dorsal horn, which in turn inhibit the function of medullary inspiratory neurons resulting in apnea. We have also shown for the first time that the influence of RSV infection on the apnea triggered by sensorineural stimulation can be reduced or even abolished with the use of humanized MAb against the viral F protein administered before or in the early phase of the infection.

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