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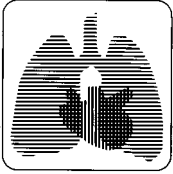
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Respiratory Syncytial Virus Causes Increased Bronchial Epithelial Permeability*

Muna M. Kilani, MD; Kamal A. Mohammed, PhD†; Najmunnisa Nasreen, PhD†; Joyce A. Hardwick, BA; Mark H. Kaplan, PhD; Robert S. Tepper, MD, PhD; and Veena B. Antony, MD†

Background: Respiratory syncytial virus (RSV)-induced diseases are mediated through active cytokines released during infection. We hypothesized that RSV infection causes bronchial epithelial monolayer permeability *in vitro* via induction of vascular endothelial growth factor (VEGF).

Methods: Human bronchial epithelial cells were infected with RSV. In some cultures, VEGF antibody was included to block VEGF response; in other cultures, palivizumab was added to block RSV infection. Permeability was assessed in real-time using electric cell-substrate impedance sensing. VEGF release was assessed using enzyme-linked immunosorbent assay. Gap formation was assessed using live cell imaging.

Results: RSV-infected cells demonstrated a decrease in the resistance of the monolayer indicating an increase in permeability; this increase was blocked with VEGF-specific antibody, and palivizumab. Intercellular gap formation developed in RSV-infected epithelial monolayers.

Conclusion: RSV increases permeability of the bronchial airway epithelial monolayer via VEGF induction. (CHEST 2004; 126:186–191)

Key words: edema; electric cell-substrate impedance sensing; respiratory syncytial virus; vascular endothelial growth factor

Abbreviations: ECIS = electric cell-substrate impedance sensing; ELISA = enzyme-linked immunosorbent assay; HBEpC = human bronchial epithelial primary cell; MOI = multiplicity of infection; RSV = respiratory syncytial virus; VEGF = vascular endothelial growth factor

Respiratory syncytial virus (RSV) is the most common cause of lower respiratory tract infections in children worldwide.¹ RSV-infected epithelial cells help to initiate antiviral immune responses and airway in-

flammation that may lead to airway obstruction.² RSV infection accounts for $\geq 70\%$ of all cases of infantile bronchiolitis, and has been linked to subsequent asthma or reactive airway disease, either directly or through a shared common predisposition.³ It is also recognized as a major cause of morbidity and mortality in immunodeficient patients.^{4,5} RSV causes an estimated 31 bronchiolitis-associated hospital admissions per year per 1,000 children < 1 year old.⁶ RSV is a labile paramyxovirus that produces a characteristic fusion of human cells in tissue culture known as the syncytial effect. Two subtypes, A and B, have been identified. Subtype B is characterized as the asymptomatic strain of the virus that the majority of the population experience. The more severe clinical illnesses involve subtype A strain, which tend to predominate in most outbreaks.⁷

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RSV affects the upper and lower respiratory tracts, but is most prevalent in lower respiratory illnesses such as pneumonia and bronchiolitis. Infection results in damage to small and medium-size bronchioles. The mechanism and signaling pathways activated by RSV that result in airway epithelial edema and inflammation are not completely understood.

RSV replicates in ciliated epithelial cells of the airway. Infection of the lower airway is associated with damage of the bronchiolar epithelium, a mononuclear inflammatory response, and plugging of the smaller bronchioles with mucus, cellular debris, fibrin strands, and DNA-like materials.⁸ Submucosal edema is also a prominent feature of RSV bronchiolitis and pneumonia.⁸ It has been assumed that the edema is due to the virus-induced epithelial damage that is characteristic of these lesions. A study⁹ has, however, demonstrated that cytokines such as vascular endothelial growth factor (VEGF) can also directly alter tissue permeability.⁹ Our laboratory has shown that VEGF can cause permeability changes in airway epithelial and mesothelial cell monolayers.^{10,11}

VEGF is a pleiotropic dimeric glycoprotein, and was first described as a tumor cell-derived molecule that increased vascular permeability.¹² VEGF is produced by a variety of cells including epithelial cells, and the lung appears to be an important source of this molecule.^{13,14} VEGF is up-regulated at sites of angiogenesis and inflammation.^{15,16} VEGF has been shown to be produced in response to RSV infection,¹⁷ and RSV bronchiolitis and pneumonia are characterized by mucosal edema.⁸ However, it is not clear if RSV infection can cause bronchial epithelial cell (HBEPc) barrier dysfunction. In this study, we demonstrate that a low multiplicity of RSV infection induces hyperpermeability of the bronchial epithelial monolayer through the release of VEGF. We hypothesize that VEGF produced as a result of RSV infection is responsible for the hyperpermeability and intercellular gap formation in bronchial airway epithelial monolayer.

MATERIALS AND METHODS

Viral Stock Preparation

RSV (A2 strain) was obtained from the American Type Culture Collection (Manassas, VA). Hep-2 cells from the American Type Culture Collection were infected with a low-input multiplicity of infection (MOI). When infection became advanced, cell supernatants were harvested, and cells were disrupted by freeze, thawing. Debris was pelleted by low-speed centrifugation. Aliquots of clarified supernatants were frozen at -70°C . Titers of infectivity of stock viruses were determined by inoculation of serial dilutions into Hep-2 cells and by quantification of plaque formation.¹⁷

Cells and Cell Culture

HBEPcs purchased from Cell Applications (San Diego, CA) were used in these studies. Serum-free bronchial epithelial growth medium with supplements were purchased from the same provider. The HBEPcs were grown and subcultured following the instructions of the supplier in 48-well plates. On the day of infection, the medium bathing the cells was aspirated and the cultures were inoculated with virus stock at different MOIs. After adsorption at 37°C for 90 min, the viral solution was removed, cells were washed with media two times, and the cells were incubated at 37° for the desired periods of time. The infected cells and control were stained for viability at all the different time points and at 48 h after infection with an MOI of 0.05, and an MOI of 0.005; 5.6% and 5.9% of the cells were dead, respectively, compared to 4.9% in the control wells. The supernatants were then collected, clarified by low-speed centrifugation, and stored at -70°C until analyzed. In some experiments, $40\ \mu\text{g}/\text{mL}$ of palivizumab (Synagis; MedImmune; Gaithersburg, MD) was added to the virus for 20 min. The HBEPcs were inoculated with the virus.

Real-time Electric Cell-Substrate Impedance Sensing

The real-time electrical resistance of both the infected and noninfected epithelial monolayers was measured by an electric cell-substrate impedance sensing system (ECIS) [Applied Biophysics; Troy, NY]. Briefly, bronchial epithelial cells were cultured on a small gold electrode ($10^{-4}\ \text{cm}^2$), and culture media was used as the electrolyte. The total electrical resistance was measured dynamically across the HBEPc monolayer. Experiments were conducted on wells that achieve 4,000 to 7,000 ohms of steady-state resistance. The HBEPcs were infected with RSV at an MOI of 0.5, 0.05, and 0.005. Some of the wells were treated with VEGF mouse antibody (PeproTech; Rocky Hill, NJ) at a concentration of $20\ \text{mg}/\text{mL}$ for 1 h prior to infection with RSV, and at 20 h after infection. Some other wells received isotype antibody (Calbiochem; San Diego, CA) as a control. The resistance was expressed by the in-phase voltage (proportional to the resistance), which was normalized to the initial voltage and expressed as a fraction of the normalized value.^{18,19} For some of the experiments, $40\ \mu\text{g}/\text{mL}$ of palivizumab was added to the viral suspension for 20 min, and the cells were inoculated with the virus. The antibody was redosed at 20 h after infection.

Measurement of VEGF by Enzyme-Linked Immunosorbent Assay

VEGF levels in RSV infected with an MOI of 0.05, and an MOI of 0.005 epithelial culture supernatants were measured by "sandwich" enzyme-linked immunosorbent assay (ELISA) [Quantikine ELISA; R&D Systems; Minneapolis, MN] as previously described.²⁰ Briefly, the samples were added to 96-well microtiter plates, which were coated with murine monoclonal antibody to VEGF. The unbound protein was washed three times, and an enzyme-linked polyclonal antibody specific to VEGF was added. The plate was washed again three times, and substrate solution was added to the wells. After 30 min of incubation, stop solution was added to each well. The amount of VEGF was determined by comparing the optical density of the samples to the standards at 450 nm using the ELISA reader.

Live Cell Imaging

HBEPcs were grown to confluence in four well-chambered glass slides (Chambered Cover Glass System; Nalge Nunc Inter-

national; Naperville, IL). HBEpCs were infected with the virus at two different concentrations (MOI of 0.005 and 0.05), and pictures were taken at 6 h, 24 h, and 48 h using differential interference contrast at a magnification of 630 ×.

Statistical Analysis

The significance of differences between experimental and control groups was tested by analysis of variance using SigmaStat statistical software (SPSS; Chicago, IL). The significance of difference between the two groups was tested by an all-pairwise multiple comparison procedure (Student-Newman-Keuls method), and $p < 0.05$ was considered significant.

RESULTS

RSV Induces HBEpC Monolayer Permeability In Vitro

When the cells attach on the bottom of the gold electrode, the electrical properties of the circuit change. The amplifier measures the resistance of the monolayer (the tightness of the junctions between cells in the monolayer); the HBEpCs had resistances starting between 4,500 ohms and 7,000 ohms for the healthy uninfected monolayers. After infection with RSV (MOI of 0.5, 0.05), the resistance began to drop 24 h after infection (Fig 1). When RSV with an MOI of 0.005 was used, the electrical resistance across the HBEpC monolayer decreased gradually with a peak decline at approximately 30 h after infection. Addition of the VEGF antibody at the beginning and at 20 h after infection blocked the fall in resistance to a

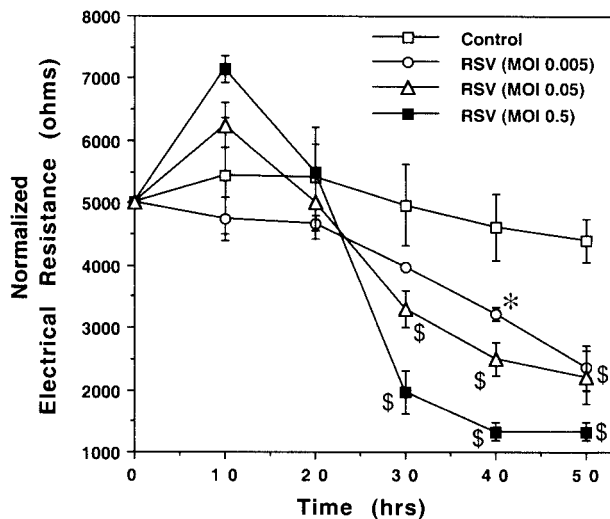


FIGURE 1. Electrical resistance across RSV-infected primary human bronchial epithelial monolayer at an MOI of 0.5, 0.05, and 0.005, and control uninfected monolayer. The x-axis represents time in hours, and the y-axis represents electrical resistance. The data points represent mean \pm SD of three separate experiments with each condition run in duplicate. * $p < 0.01$ compared to control. $^{\$}p < 0.001$ compared to control.

level similar to the control (Fig 2). Inclusion of an isotype antibody to irrelevant antigen had no effect on the RSV-induced barrier dysfunction. Palivizumab treatment was able to decrease the fall in resistance at the 30-h and 40-h time points after infection, but ultimately at 50 h after infection the electrical resistance across the monolayer reached levels similar to the RSV-infected cells without the antibody (Fig 3).

RSV-Induced VEGF Production in HBEpCs In Vitro

RSV enhanced VEGF release in HBEpCs in a time-dependent manner. Infection of HBEpCs with RSV increased the VEGF production at 48 h when compared to uninfected control cultures, and this was statistically significant. There was some increase in VEGF release at 24 h of infection; however, it was significant only when compared to the negative control. The VEGF levels in controls was 492 ± 71 pg/mL, 545 ± 208 pg/mL, and 559 ± 20 pg/mL at 6 h, 24 h, and 48 h of incubation, respectively; in RSV-infected HBEpCs, the VEGF levels were 625 ± 40 pg/mL, 885 ± 170 pg/mL, and 985 ± 33 pg/mL, at 6 h, 24 h, and 48 h after infection, respectively (Fig 4).

Palivizumab significantly decreased the production of VEGF when used with RSV with an MOI of 0.005 at 48 h after infection; the VEGF levels were

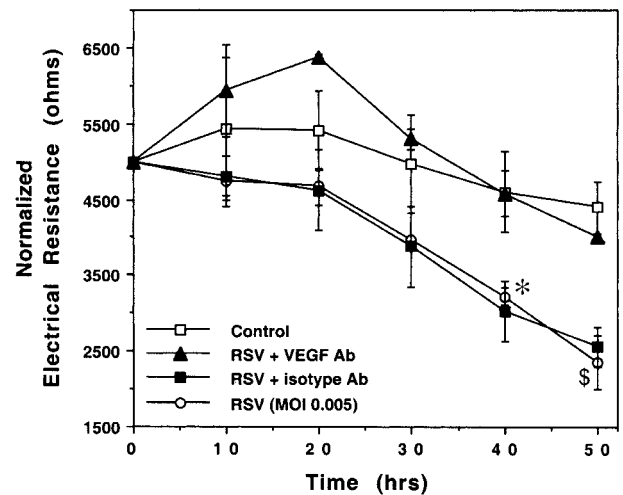


FIGURE 2. Electrical resistance in RSV infected (MOI of 0.005) uninfected control primary HBEpC monolayer in presence of VEGF antibody (Ab). VEGF antibody was added to the cells at a concentration of $20 \mu\text{g/mL}$ at zero and 20 h. In parallel cultures, the isotype antibody was added at the same dose and time. The data points represent mean \pm SD of three separate experiments with each condition run in duplicate. The addition of the VEGF antibody recovered the decrease in resistance to the extent of control levels. * $p < 0.01$ compared to control. $^{\$}p < 0.001$ compared to control.

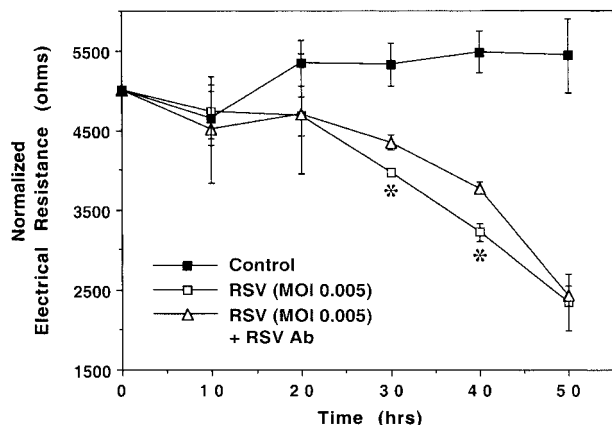


FIGURE 3. Electrical resistance in RSV infected (MOI of 0.005) uninfected control primary HBEpC monolayer in the presence of palivizumab. RSV antibody (palivizumab) was added to the cells at a concentration of 40 $\mu\text{g/mL}$ at zero and 20 h. The data points represent mean \pm SD of three separate experiments with each condition run in duplicate. The addition of Palivizumab (monoclonal antibody to RSV) recovered the decreased resistance to some extent after 30 h. * $p < 0.001$ compared to control. See Figure 2 legend for expansion of abbreviation.

587 \pm 36 pg/mL, 699 \pm 40 pg/mL, and 822 \pm 32 pg/mL at 6 h, 24 h, and 48 h after infection. When palivizumab was added to RSV with an MOI of 0.05, there was no significant change in VEGF production (data not shown).

RSV Induces Gap Formation in HBEpCs

RSV induced gap formation in HBEpCs in a concentration- and a time-dependent manner (Fig 5). RSV with an MOI of 0.005 induced gap formation 48 h after infection. The cells infected with an MOI of 0.05 RSV showed no gap formation at 6 h; however, there was gap formation between cells at 24 h. After 48 h after infection, gap formation was obvious at both concentrations used and correlated with the decrease in resistance revealed by ECIS.

DISCUSSION

Our study demonstrated that VEGF release in RSV infection contributed directly to the increase in the permeability of the epithelial monolayer and gap formation. We used the ECIS technique and direct visualization of the live cells, and were able to detect the decrease in resistance of the monolayer and visualize the gap formation. Palivizumab, the monoclonal antibody against RSV, blocked the decrease in electrical resistance of the epithelial monolayer to some extent at the later time points. We also demonstrated a decrease in the VEGF production after addition of the antibody.

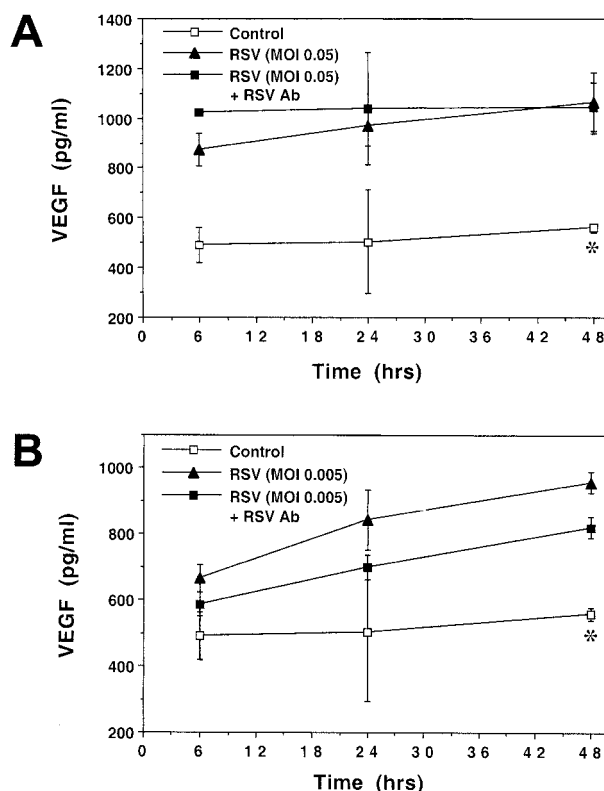


FIGURE 4. RSV-induced VEGF release in HBEpC. HBEpCs were infected with RSV of an MOI 0.05 (top, A), MOI 0.005 (bottom, B), and the supernatants were collected at 6 h, 24 h, and 48 h as described in the "Materials and Methods" section. Palivizumab (monoclonal antibody to RSV) was co-cultured with the virus for 20 min before infection. Palivizumab was added again to the culture 20 h after infection. * $p < 0.001$ compared to control. See Figure 2 legend for expansion of abbreviation.

RSV infection continues to cause significant morbidity and mortality, and is a burden on the health-care system. The use of a monoclonal antibody against RSV helped in decreasing the number of hospital admissions infants with RSV infection, who were considered high risk for infection¹⁹; however, no vaccine against RSV infection is available. Inflammation is an important feature of RSV infection. In RSV infection, plasma and inflammatory cells move into the interstitium, the bronchial walls, and into the airway lumen. These exudates cause airway wall thickening and contribute to the formation of viscous mucus and airway plugging that are characteristic of RSV bronchiolitis.⁸ Thus, it is possible that alterations in airway permeability contribute to the obstruction and the structural changes seen in the airways of infants who have had several RSV infections. Mediators released during inflammation influence both the epithelial and vascular compartments. VEGF has been previously shown to be produced during RSV infection, and it has been assumed that its effects on the vascular tissue alone contributed to

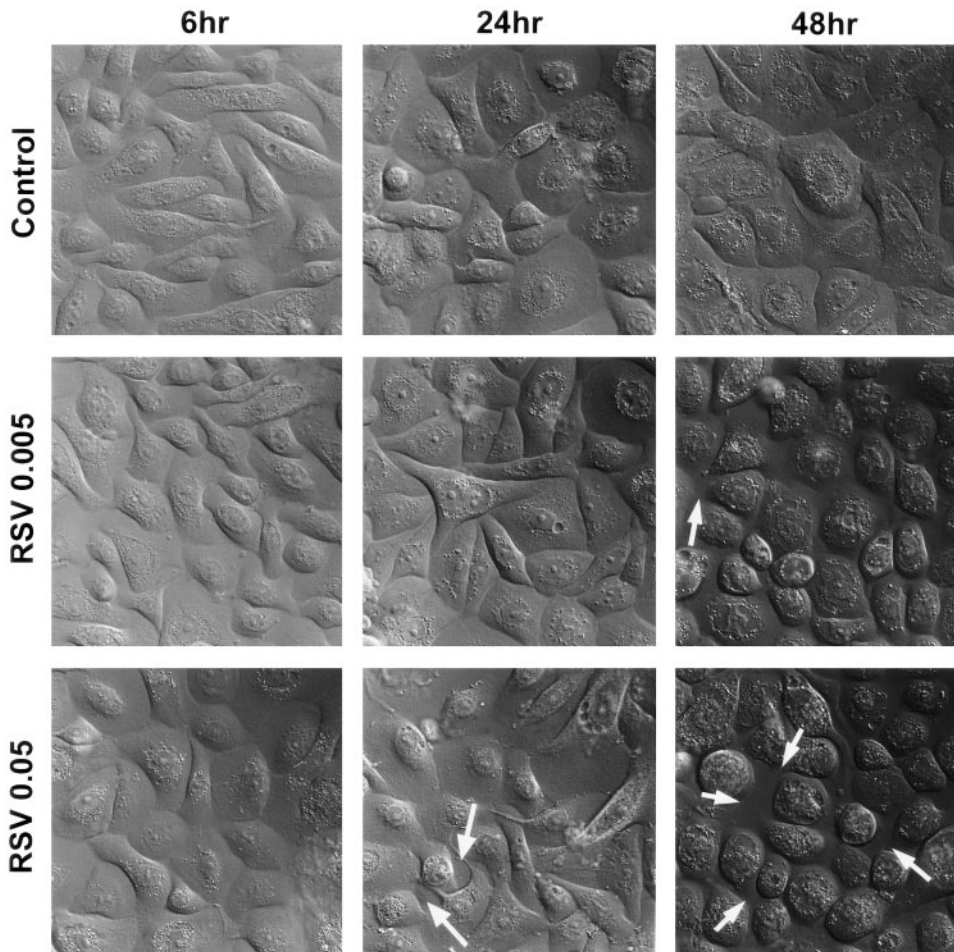


FIGURE 5. RSV-induced gap formation in HBEpC. HBEpCs were infected with RSV at an MOI of 0.005, and 0.05. Pictures were taken at 6 h, 24 h, and 48 h after infection using differential interference contrast at a magnification of, 630. Gap formation was noted in the MOI 0.05 at 24 h, and in both concentrations at 48 h. These images are single representatives of three independent experiments.

edema formation.¹⁷ VEGF is recognized to cause vascular leakage by changes in the endothelium, and it can cause permeability changes in mesothelial and epithelial cell monolayers.^{8,9} Our results show that VEGF causes tissue edema in RSV infection due not only to changes in the vascular permeability, but also causes intercellular gap formation and permeability in the airway epithelium.

The respiratory epithelium is critically important for protecting the underlying tissue against environmental pollutants and pathogens. The integrity of the epithelial monolayer depends on cell-cell adhesion, and VEGF stimulation can affect cell-cell adhesion by rapid tyrosine phosphorylation of cadherins and other adhesion molecules in endothelial tissue.²⁰ In airway epithelial cells, the expression of cadherins and catenins are modulated by inflammatory cytokines.²¹ VEGF production seems to be at least in part specific to RSV infection. Lee et al¹⁷ demonstrated that rhinovirus

(another respiratory virus) was unable to stimulate VEGF production. In the same study,¹⁷ the authors found children with RSV infection had higher levels of VEGF in their nasal secretions compared to children with influenza infection, or control children without a respiratory infection. Palivizumab decreased RSV-mediated VEGF release in HBEpCs, suggesting that the VEGF released was specific to RSV infection. VEGF antagonism reduces edema formation and damage after ischemia-reperfusion injury.²² In the present study, VEGF antagonism reversed the permeability changes in the epithelial monolayer after RSV infection.

The current study is interesting because we demonstrate that in RSV infection, the virus induces VEGF release by the bronchial epithelium. The released VEGF decreases epithelial resistance, increases epithelial cell-cell gap formation, and leads to bronchial hyperpermeability.

REFERENCES

- 1 Dodge R, Martinez FD, Cline MG, et al. Early childhood respiratory symptoms and the subsequent diagnosis of asthma. *J Allergy Clin Immunol* 1996; 98:48–54
- 2 Gern JE. Mechanisms of virus-induced asthma. *J Pediatr* 2003; 142:S9–S13
- 3 Openshaw PJ, Dean GS, Culley FJ. Links between respiratory syncytial virus bronchiolitis and childhood asthma: clinical and research approaches. *Pediatr Infect Dis J* 2003; 22:S58–S64
- 4 Dowell SF, Anderson LJ, Gary HE Jr, et al. Respiratory syncytial virus is an important cause of community-acquired lower respiratory infection among hospitalized adults. *J Infect Dis* 1996; 174:456–462
- 5 Falsey AR, Cunningham CK, Barker WH, et al. Respiratory syncytial virus and influenza A infections in the hospitalized elderly. *J Infect Dis* 1995; 172:389–394
- 6 Shay DK, Holman RC, Newman RD, et al. Bronchiolitis-associated hospitalizations among US children, 1980–1996. *JAMA* 1999; 282:1440–1446
- 7 Bar-on ME, Zanga JR. Bronchiolitis. *Prim Care* 1996; 23: 805–819
- 8 Hayden FG, Richman DD, Whitley RJ, eds. *Clinical virology*. New York, NY: Churchill Livingstone, 1997
- 9 Roberts WG, Palade GE. Neovasculature induced by vascular endothelial growth factor is fenestrated. *Cancer Res* 1997; 57:765–772
- 10 Sriram PS, Mohammed KA, Nasreen N, et al. Adherence of ovarian cancer cells induces pleural mesothelial cell (PMC) permeability. *Oncol Res* 2002; 13:79–85
- 11 Antony AB, Tepper RS, Mohammed KA. Cockroach extract antigen increases bronchial airway epithelial permeability. *J Allergy Clin Immunol* 2002; 110:589–595
- 12 Neufeld G, Cohen T, Gengrinovitch S, et al. Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J* 1999; 13:9–22
- 13 Maniscalco WM, Watkins RH, Finkelstein JN, et al. Vascular endothelial growth factor mRNA increases in alveolar epithelial cells during recovery from oxygen injury. *Am J Respir Cell Mol Biol* 1995; 13:377–386
- 14 Berse B, Brown LF, Van de Water L, et al. Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors. *Mol Biol Cell* 1992; 3:211–220
- 15 Brown LF, Detmar M, Claffey K, et al. Vascular permeability factor/vascular endothelial growth factor: a multifunctional angiogenic cytokine. *EXS* 1997; 79:233–269
- 16 Ferrara N, Keyt B. Vascular endothelial growth factor: basic biology and clinical implications. *EXS* 1997; 79: 209–232
- 17 Lee CG, Yoon HJ, Zhu Z, et al. Respiratory syncytial virus stimulation of vascular endothelial cell growth factor/vascular permeability factor. *Am J Respir Cell Mol Biol* 2000; 23:662–669
- 18 Lundien MC, Mohammed KA, Nasreen N, et al. Induction of MCP-1 expression in airway epithelial cells: role of CCR2 receptor in airway epithelial injury. *J Clin Immunol* 2002; 22:144–152
- 19 Mohammed KA, Nasreen N, Hardwick J, et al. Bacterial induction of pleural mesothelial monolayer barrier dysfunction. *Am J Physiol Lung Cell Mol Physiol* 2001; 281:L119–L125
- 20 Mohammed KA, Nasreen N, Ward MJ, et al. Induction of acute pleural inflammation by *Staphylococcus aureus*: I. CD4+ T cells play a critical role in experimental empyema. *J Infect Dis* 2000; 181:1693–1699
- 21 Carayol N, Campbell A, Vachier I, et al. Modulation of cadherin and catenins expression by tumor necrosis factor- α and dexamethasone in human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 2002; 26:341–347

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