A Respiratory Syncytial Virus (RSV) Anti-G Protein F(ab')2 Monoclonal Antibody Suppresses Mucous Production and Breathing Effort in RSV rA2-line19F-Infected BALB/c Mice

Seyhan Boyoglu-Barnum, Emory University
Kelsey A. Gaston, Emory University
Sean O. Todd, Emory University
Cemil Boyoglu, Emory University
Tatiana Chirkova, Emory University
Thomas R. Barnum, University of Georgia, Athens
Patricia Jorquera, University of Georgia, Athens
Lia M. Haynes, Centers for Disease Control and Prevention
Ralph A. Tripp, University of Georgia, Athens
Martin L. Moore, Emory University

Only first 10 authors above; see publication for full author list.

Journal Title: Journal of Virology
Volume: Volume 87, Number 20
Publisher: American Society for Microbiology | 2013-10, Pages 10955-10967
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1128/JVI.01164-13
Permanent URL: http://pid.emory.edu/ark:/25593/ft6pg

Final published version: http://jvi.asm.org/content/87/20/10955

Copyright information:
© 2013, American Society for Microbiology. All Rights Reserved.

Accessed February 13, 2020 8:12 AM EST
A Respiratory Syncytial Virus (RSV) Anti-G Protein F(\(ab{'}\))\(_2\) Monoclonal Antibody Suppresses Mucous Production and Breathing Effort in RSV rA2-line19F-Infected BALB/c Mice

Seyhan Boyoglu-Barnum, Kelsey A. Gaston, Sean O. Todd, Cemil Boyoglu, Tatiana Chirkova, Thomas R. Barnum, Patricia Jorquera, Lia M. Haynes, Ralph A. Tripp, Martin L. Moore, Larry J. Anderson

Emory University Department of Pediatrics and Children’s Healthcare of Atlanta, Atlanta, Georgia, USA; Division of Viral Diseases, NCIRD, CDC, Atlanta, Georgia, USA; University of Georgia Department of Infectious Diseases, Animal Health Research Center, Athens, Georgia, USA; University of Georgia Odum School of Ecology, Athens, Georgia, USA

Respiratory syncytial virus (RSV) belongs to the family Paramyxoviridae and is the single most important cause of serious lower respiratory tract infections in young children, yet no highly effective treatment or vaccine is available. Increased airway resistance and increased airway mucin production are two manifestations of RSV infection in children. RSV rA2-line19F infection induces pulmonary mucous production and increased breathing effort in BALB/c mice and provides a way to assess these manifestations of RSV disease in an animal model. In the present study, we investigated the effect of prophylactic treatment with the F(\(ab{'}\))\(_2\) form of the anti-G protein monoclonal antibody (MAb) 131-2G on disease in RSV rA2-line19F-challenged mice. F(\(ab{'}\))\(_2\) 131-2G does not affect virus replication. It and the intact form that does decrease virus replication prevented increased breathing effort and airway mucin production, as well as weight loss, pulmonary inflammatory-cell infiltration, and the pulmonary substance P and pulmonary Th2 cytokine levels that occur in mice challenged with this virus. These data suggest that the RSV G protein contributes to prominent manifestations of RSV disease and that MAb 131-2G can prevent these manifestations of RSV disease without inhibiting virus infection.

Received 30 April 2013 Accepted 12 July 2013
Published ahead of print 24 July 2013
Address correspondence to Larry J. Anderson, larry.anderson@emory.edu.
Copyright © 2013, American Society for Microbiology. All Rights Reserved.
which has been reported to be present in RSV-specific T cells from infants with RSV bronchiolitis (29).

Since increased airway resistance and mucous production are associated with RSV infection in humans (30–32), their induction by RSV A2-line19F provides additional clinically relevant outcomes to study in mice (25, 33). In this study, we chose to determine if the F(ab') 2 form of MAb 131-2G can block these features of disease with rA2-line19F infection and, consequently, determine if the G protein likely contributes to these manifestations of RSV disease. We used the F(ab') 2 form of 131-2G to eliminate decreased virus replication as a reason for the observed effects. Our results show that prophylaxis with the F(ab') 2 form of MAB 131-2G, as well as the intact form, decreased airway mucin production, pulmonary cellular infiltration, pulmonary substance P levels, and breathing effort. These data suggest that prevention of increased mucous production and airway dysfunction can be added to the potential benefits of binding G protein with antibody against the central conserved region of G protein.

MATERIALS AND METHODS

Animals. Animal studies were performed according to a protocol approved by the Emory University (Atlanta, GA) Institutional Animal Care and Use Committee. Four- to 6-week-old, specific-pathogen-free female BALB/c mice (Charles River Laboratory, Wilmington, MA) were used in all experiments. Mice were housed in microisolator cages and fed sterilized water and food ad libitum. In study 1, mice were anesthetized by intraperitoneal administration of tribromoethanol (Avertin; 2% 2,2,2-trifluoroethanol in sterilized water and food) and killed by cervical dislocation. After dissection of the lungs with forceps, lungs were cut into small fragments and treated with 100 units/ml Dispase II (Worthington, Freehold, NJ) for 1 h at 37°C. The lung fragments were homogenized with a Dounce homogenizer and the homogenates were centrifuged at 10,000 × g for 15 min at 4°C. The supernatant was collected and the pellets were rehomogenized and centrifuged. The supernatants were combined and centrifuged at 100,000 × g for 1 h at 4°C. The pellets were discarded and the supernatants were used for ELISAs.

FIG 1 Experimental schedule for animal studies. Days are numbered relative to the day of RSV challenge.

Virus preparation. RSV A2 or rA2-line19F was propagated in HEp-2 cells. RSV A2 was included in some experiments to enhance comparisons of the results of this study to those of earlier studies with rA2-line19F. Briefly, 5 days after inoculation of HEp-2 cells with RSV A2 or rA2-line19F at a multiplicity of infection (MOI) of 0.01, loosely capped flask containing the cells were transferred to a –80°C freezer overnight. After thawing at 4°C, cells were scraped down and the cell lysates were transferred to 50-ml conical tubes. The cell debris was removed by centrifugation at 3,000 rpm for 7 min at 4°C. The supernatants were pooled and purified by centrifugation on a sucrose cushion (20% sucrose) at 16,000 × g for 4 h. After centrifugation, the supernatant was removed and the pellet was dissolved in serum-free MEM. The viral pools were divided into aliquots, quick-frozen in liquid nitrogen, and stored at −80°C until they were used.

Virus infectivity titers were determined by microinfectivity assays as previously described (34). Briefly, serial dilutions of the virus preparation in minimal essential medium supplemented with fetal bovine serum (Fisher, Pittsburgh, PA), 2 mM l-glutamine (Gibco, Grand Island, NY), and 5,000 units/ml penicillin-streptomycin (Gibco, Grand Island, NY) were inoculated onto subconfluent HEP-2 cells in 96-well microtiter plates. After 2 h of adsorption at 37°C, 180 μl of tissue culture media was added, and 5 days later, the cells were fixed with 80% acetone in 1× phosphate-buffered saline (PBS). Replication of virus was determined by RSV enzyme-linked immunosorbent assay (ELISA) using plates with the acetone-fixed RSV-infected HEP-2 cells. The plates were blocked with 5% bovine serum albumin (BSA) in 1× PBS for 1 h at 37°C, and then goat anti-RSV antibody (Millipore, Billerica, MA) was added and the plates were incubated for 1 h at 37°C. The plates were washed with 1× PBS containing 0.05% Tween and then incubated for 1 h at 37°C with peroxidase-conjugated donkey anti-goat antibody (Jackson Immunoresearch, West Grove, PA). After a second washing, color was developed with o-phenylenediamine (OPD) substrate (Sigma-Aldrich) as indicated by the manufacturer. The enzyme-substrate reaction was terminated by the addition of a sulfuric acid solution, and absorbance was measured at a wavelength of 450 nm. The infectivity titer was determined using the Reed and Muench method (35).

F(ab')2 fragment preparation and treatment. Monoclonal antibody 131-2G F(ab')2 fragments were generated by pepsin digestion (Sigma-Aldrich). Briefly, purified MAb 131-2G was digested with porcine pepsin overnight at 37°C, and digested MAb was passed through a protein G Sepharose column (GE Healthcare, Alpharetta, GA) to eliminate Fc fragments and undigested antibodies. Purified F(ab')2 fragments were dialyzed and concentrated using a Centricon spin column (Millipore, Temecula, CA) with a 30-kDa cutoff. The purity of the F(ab')2 fragments was determined by SDS-PAGE (Bio-Rad, Hercules, CA) under nonreducing conditions. The protein concentration was determined by bicinchoninic acid (BCA) protein assay (Pierce Protein Research Products, Rockford, IL). Endotoxin concentrations were determined using a Limu-
lue amebocyte lysate chromogenic endpoint assay in accordance with the manufacturer’s instructions (Lonza, Atlanta, GA). Intact 131-2G was included in some experiments to determine if it had effects on rA2-line19F disease similar to those of F(ab')2, 131-2G and if it would also decrease replication of rA2-line19F in mice.

**Quantification of lung viral load.** Pulmonary viral load replication was assessed by measuring infectious virus in homogenized lung tissue. To homogenize the lungs, we used a BeadBeater (Biospec Products, Bartlesville, OK). Two-milliliter-deep 96-well plates (Axygen Scientific, Union City, CA) were loaded with 1 ml of zirconium beads (Biospec Products, Bartlesville, OK), sealed with a rubber lid (Axiomat; Axygen Scientific, Union City, CA), and autoclaved. Sterile basal MEM (500 μl) was added to each well, and the plates were briefly centrifuged and then kept on ice. For each mouse, the left lung lobe was harvested, weighed, cut into four to five pieces, and placed into a well of a 2-ml-deep 96-well plate on ice. An additional 400 μl of basal MEM was added to each well, and the plates were sealed with the Axiomat lids. The plates were shaken in the BeadBeater for 1 min, followed by a 1-min plunge in an ice and water slurry. This was repeated 12 times for a total of 12 -1 min homogenizations. The plates were centrifuged at 2,000 rpm for 5 min at 4°C. Lung supernatants were divided into aliquots; one aliquot was used for virus infectivity titration and the remaining aliquots were stored at −80°C for future studies. Virus infectivity titers were determined as described above in the section “Virus Preparation.”

**Viral RNA levels were determined by RSV real-time PCR.** Total RNA was extracted from bronchoalveolar lavage (BAL) fluid using a Qiagen total-RNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions and stored at −80°C. Quantitative real-time PCR was performed by using an AgPath-ID one-step reverse transcription (RT)-PCR kit (Applied Biosystems, Foster City, CA) and an ABI 7700 sequence detection system (PE Applied Biosystems, Foster City, CA). Thermal cycling conditions included 45°C for 2 min and 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 s and 55°C for 2 min for denaturing and annealing, respectively. The primers and probes for the RSV M (M) gene (forward primer, 5'-GGC AAA TAT GGA AAC ATA GCC GAA-3'; reverse primer, 5'-TCT TCT AGG ACA TTC TAY TGA ACA G-3'; probe, 5'-6-carboxyfluorescein (FAM)-TGT CCG TCT TCT TCT AGG CCC TCG TC-3' black hole quencher 1 (BHQ-1)-3' (36) were obtained from Integrated DNA Technologies (IDT) (Corvalle, IA). Threshold cycles (Ct) for each sample were calculated. Assays were performed in duplicate for each experiment, and they were repeated for three different sets of mice.

**BAL leukocyte specimens.** Mice were anesthetized and euthanized by exsanguination after severing of the left axillary artery. Leukocytes were harvested by lavaging the lungs 3 times using 1 ml sterile 1X phosphate-buffered saline (PBS) for each wash. The BAL cells were stained for extracellular markers using microcultture staining according to the protocol described by Tripp et al. (12). Briefly, BAL cells were blocked in Fc blocker (anti-CD16/32) in 1× PBS with 1% bovine serum albumin for 15 min at 4°C and then stained for 30 min at 4°C in the dark with the appropriate combinations of anti-CD3 (17A2) (T cells), anti-CD4 (GK1.5) (T cells), anti-CD8 (53-67.2) (T cells), anti-CD45RB/B220 (RA3-6B2) (B cells), anti-CD11b (M1/70) (macrophages, dendritic cells, and monocytes), anti-mouse Ly-6G/Gr-1 (RB6-8C5) (polymorphoenuclear cells [PMNs]), anti-mouse CD49b/integrin alpha 2 (DX5) (NK and NK T cells), and mouse isotype antibody controls (all from eBiosciences, San Diego, CA) diluted in staining buffer. The distributions and patterns of cell surface markers were determined for 10,000 lymphocyte-gated events analyzed by staining with FITC-anti-CD4, PerCPCy5.5-anti-CD8, and PE-anti-CD11c. The data were analyzed using FlowJo software (TreeStar, Ashland, OR).

**Quantitation of substance P.** Substance P (SP) levels in cell-free BAL supernatant were analyzed by using a competitive ELISA kit (Cayman Chemical, Ann Arbor, MI) in accordance with the manufacturer’s instructions and as previously described (37). The assay is based on the competition between free SP and an SP tracer for a limited number of SP-specific binding sites. The percent sample bound/maximum bound was calculated, and the SP concentration in each sample was determined based on the percent standard bound/maximum bound versus the standard SP concentration. The intra- and interassay coefficients of variation are 10%.

**Pulse oximetry.** Pulsus paradoxus is an exaggeration of normal variation in the pulse volume with respiration that can be caused by labored breathing and the associated increased negative and positive intrathoracic pressures with inspiration and expiration, respectively. The breath-associated difference in pulse volume (difference between systole and diastole) was assessed by measuring the absorbance of light passing through tissue with a rodent pulse oximeter (Mousse; Starr Life Sciences Corp., Oakmont, PA). The difference in light absorbance between systole and diastole was used to calculate changes in the effective path length of light passing through pulsating vessels or arterioles as an indicator of pulse volume, and differences in pulse volume between inspiration and expiration were estimated and reported as breath distortion in microns (38). For these measurements, mice were anesthetized using an isoflurane (2.5%)-oxygen (2 liters/min) mixture provided by an anesthesia machine (VetEquip, Pleasanton, CA). Mice were anesthetized five at a time in a Plexiglas box connected to the anesthesia machine. One mouse at a time was removed from the Plexiglas box and placed on its back on a pad with its nose in an isoflurane-oxygen nosecone connected to the anesthesia machine. The anesthetized mouse’s feet were restrained to a pad with tape, and the Mousse sensor was applied to the mouse’s thigh. The mouse was covered with a cloth to reduce ambient light, and its arterial O2 saturation, heart rate, pulse rate, pulse distension, and breath distension were recorded approximately every 0.1 s (Mousse software, version 4.0) for 1 to 5 min. Only time points in which all of these physiological parameters were successfully measured were included in analyses, giving 30 to 2 min of measurements per mouse. The average recorded breath-associated change in vessel wall distension (in microns) was used as the value for each mouse. Data were analyzed using Microsoft Excel and presented in microns.

**Determination of Muc5AC protein expression.** Mucin-5AC protein concentrations in lung tissues were assayed with a mouse mucin-5 subtype AC ELISA kit (USCN Life Science, Inc., Wuhan, China) used according to the manufacturer’s protocol. Briefly, an aliquot of homogenized lung tissue was thawed, and the total protein concentration was determined by a BCA protein assay kit (Thermo Scientific, Rockford, IL). Muc5AC standards and lung homogenates were added to designated microtiter plate wells precoated with anti-Muc5AC antibody. After 2 h of incubation at 37°C, biotin-conjugated polyclonal antibody was added and the wells were incubated for 1 h at 37°C. Next, avidin conjugated to horseradish peroxidase was added and the wells were incubated for 1 h at 37°C. After the wells were washed, color was developed with TMB substrate solution (3,3',5,5'-tetramethylbenzidine). The enzyme-substrate reaction was terminated by the addition of a sulfuric acid solution, and absorbance was measured at a wavelength of 450 nm. The concentration of Muc5AC in the samples was determined by comparing the optical density (OD) of the samples to the standard curve generated and expressed as a fold value.

**Histopathology.** Lungs were fixed in 10% formalin for 1 h, followed by washing with 70% ethanol and embedding in paraffin blocks. Five-micrometer sections of lung tissue were deparaffinized in xylene, rehydrated through a graded series of ethanol washes, and stained with a periodic acid-schiff (PAS) kit (Sigma-Aldrich, St. Louis, MO) to assess intracellular pulmonary mucin levels. PAS-stained slides were digitally scanned using a Hamamatsu NanoZoomer 2.0-HT slide scanner (Meyer Instruments, Houston, TX) with a 20× objective and analyzed using ImageJ software. Fifteen to 20 fields (20× magnification) per tissue section were examined.

For immunohistochemistry staining, deparaffinized sections were sequentially incubated at room temperature with blocking serum for 30 min; primary antibody, i.e., anti-Muc5AC (clone 45MI) (Santa Cruz, Del-
las, TX), at 4°C overnight; biotinylated secondary antibody (Santa Cruz, South Carolina) for 30 min; 3% H2O2 for 5 min to eliminate endogenous hydrogen peroxidase activity; and horseradish peroxidase-streptavidin for 30 min. Final visualization of antigen was achieved by incubating sections with a diaminobenzidine-H2O2 solution for 5 min until the desired stain intensity developed. The sections were digitally scanned using a Hamamatsu NanoZoomer 2.0-HT slide scanner with a 20× objective and analyzed using ImageJ software. Fifteen to 25 fields in the tissue sections were analyzed.

For immunofluorescence staining, deparaffinized sections were sequentially incubated at room temperature with avidin-biotin blocking (Vector Laboratories, Burlingame, CA) for 30 min; primary antibody, i.e., goat polyclonal anti-RSV (Millipore, Burlingame, CA), for 1 h at room temperature; biotinylated rabbit anti-goat secondary antibody (Vector Laboratories) for 30 min; and fluorescein avidin DCS (Vector Laboratories) for 10 min in the dark. The slides were mounted with ProLong Gold antifade reagent with DAPI (4′,6-diamidino-2-phenylindole; Invitrogen, Grand Island, NY) at room temperature. The sections were visualized with an Amg Evos® fl digital inverted fluorescence microscope (Fisher Scientific, Pittsburgh, PA) with a 20× objective and analyzed using ImageJ software. Fifteen to 20 fields (20× magnification) per tissue section were examined.

**Multiplex cytokine analysis.** Cytokine levels in lung homogenates were normalized to the protein present (in milligrams) in cell-free preparations of lung supernatants as measured by BCA assay used according to the manufacturer’s instructions (Thermo Scientific, Rockford, IL). The lung homogenates were centrifuged, and the cell-free supernatants were tested by multiplex Luminex assays for IL-13, IL-4, IL-5, monocyte chemotactic protein 1 (MCP-1), interferon-inducible protein 10 (IP-10), and IL-12 (Invitrogen, Valencia, CA) according to the manufacturer’s instructions (mouse cytokine 20-plex panel; Invitrogen, Valencia, CA) using an xMap 200 system (Invitrogen). The concentration of each cytokine was determined by comparison to the standard curve according to the manufacturer’s instructions. The threshold of detection was 5 pg/ml.

**Statistical analyses.** Unless otherwise indicated, groups were compared by one-way analysis of variance (ANOVA) and post hoc Tukey’s honestly significant difference (HSD) test (P < 0.05). A P value of ≤0.05 was considered statistically significant. All statistical analyses were performed using the statistical package R (R Developmental Core Team 2012). Data are shown as means ± standard errors of the means (SEMs).

**RESULTS**

**Kinetics of rA2-line19F infection.** The effects of rA2-line19F and A2 challenges (1 × 10^6 TCID_{50}) on weight loss, viral load, pulmonary inflammation, and pulsus paradoxus were determined on days 1, 3, 5, and 8. Both A2 and rA2-line19F infections caused noticeable weight loss compared to mock-challenged mice, with the peak difference on day 5 postinfection (p.i.) (P < 0.05) (Fig. 2A). rA2-line19F caused greater weight loss, but the differences were not significant. Both viruses showed the peak virus titer in the lungs at day 5 p.i., with rA2-line19F giving a 0.42 ± 0.12 log TCID_{50} higher titer than A2 (Fig. 2B) (P = 0.001). The kinetics of pulmonary inflammation paralleled the titers of virus in the lungs, with peak values on day 5 p.i. rA2-line19F-infected mice showed a
significantly greater number ($P < 0.001$) of inflammatory cells in the lung than A2-infected mice (Fig. 2C). rA2-line19F-infected mice had a 37.37% ± 16.13% increase in cell numbers in BAL fluid at day 5 p.i. Note also the difference in the types of cells in the BAL fluid (Table 1). For example, on day 5 p.i., relative to A2-infected mice, rA2-line19F-infected mice had a 74.98% ± 7.18% increase in CD3$^+$ CD45R/B220$^+$ (RA3-6B2)$^+$ cells (B cells) and a 66.99% ± 8.42% increase in CD3$^-$ CD11b$^+$ (M1/70)$^+$ cells (macrophages, dendritic cells, and monocytes) compared to a 23.34% ± 4.28% increase in CD3$^-$ Ly-6G/Gr-1$^+$ (RB6-8C5)$^+$ cells (polymorphonuclear cells), a 54.26% ± 11.37% increase in CD3$^-$ CD49b/integrin alpha 2 (DX5)$^+$ cells (NK cells), and a 47.45% ± 14.18% increase in CD8 (53.6.7)$^+$ cells (T cells), and a 56.62% ± 11.20% increase in CD4 (GK1.5)$^+$ cells. By contrast, on day 8 p.i. the most prominent increase in a BAL cell type for rA2-line19F-infected mice was the 78.30% ± 6.59% increase in CD8 (53.6.7)$^+$ cells (T cells). Pulsus paradoxus, or breathing effort, was used as our indicator of lung dysfunction, and similar to those in a previous study (25), rA2-line19F-challenged mice had a significant increase in breathing effort values compared to those for A2- and mock-challenged mice, with the peak increase on day 8 p.i. (Fig. 2D). Based on these data, we used day 5 p.i. to assess the effect of F(ab$^\prime$)$_2$ 131-2G MAb prophylaxis on weight loss, pulmonary viral load, and inflammation and day 8 p.i. to assess the effect on mucous production and breathing effort.

### Table 1: Pulmonary leukocyte trafficking after rA2-line19F and A2 RSV infections$^a$

<table>
<thead>
<tr>
<th>Day postinfection</th>
<th>Phenotype</th>
<th>Mean total no. of A2 cells ± SE ($10^3$)</th>
<th>Mean total no. of rA2-line19F cells ± SE ($10^3$)</th>
<th>% increase, A2 to rA2-line19F</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CD3</td>
<td>3.96 ± 0.57</td>
<td>4.96 ± 0.39**</td>
<td>18.90 ± 7.13</td>
</tr>
<tr>
<td></td>
<td>CD4</td>
<td>0.22 ± 0.05</td>
<td>0.35 ± 0.09</td>
<td>33.77 ± 16.89</td>
</tr>
<tr>
<td></td>
<td>CD8</td>
<td>0.24 ± 0.02</td>
<td>0.49 ± 0.08***</td>
<td>49.53 ± 5.65</td>
</tr>
<tr>
<td></td>
<td>B cell</td>
<td>2.42 ± 0.50</td>
<td>3.61 ± 1.31</td>
<td>27.67 ± 8.60</td>
</tr>
<tr>
<td></td>
<td>Macrophage</td>
<td>4.95 ± 0.64</td>
<td>8.63 ± 0.94***</td>
<td>42.01 ± 9.54</td>
</tr>
<tr>
<td></td>
<td>PMN</td>
<td>3.15 ± 0.72</td>
<td>4.76 ± 0.94**</td>
<td>40.62 ± 9.64</td>
</tr>
<tr>
<td></td>
<td>NK</td>
<td>1.86 ± 0.75</td>
<td>3.71 ± 1.14**</td>
<td>49.86 ± 9.30</td>
</tr>
<tr>
<td>3</td>
<td>CD3</td>
<td>1.36 ± 0.61</td>
<td>5.33 ± 0.45***</td>
<td>73.55 ± 13.58</td>
</tr>
<tr>
<td></td>
<td>CD4</td>
<td>0.20 ± 0.14</td>
<td>1.36 ± 0.35***</td>
<td>83.81 ± 13.45</td>
</tr>
<tr>
<td></td>
<td>CD8</td>
<td>0.16 ± 0.06</td>
<td>0.94 ± 0.09***</td>
<td>83.05 ± 6.07</td>
</tr>
<tr>
<td></td>
<td>B cell</td>
<td>1.94 ± 0.76</td>
<td>5.50 ± 0.57***</td>
<td>63.11 ± 17.94</td>
</tr>
<tr>
<td></td>
<td>Macrophage</td>
<td>6.57 ± 1.75</td>
<td>10.23 ± 1.31***</td>
<td>36.09 ± 14.15</td>
</tr>
<tr>
<td></td>
<td>PMN</td>
<td>0.35 ± 0.31</td>
<td>0.66 ± 0.12</td>
<td>41.07 ± 10.01</td>
</tr>
<tr>
<td></td>
<td>NK</td>
<td>2.04 ± 0.70</td>
<td>4.71 ± 0.22**</td>
<td>56.77 ± 19.98</td>
</tr>
<tr>
<td>5</td>
<td>CD3</td>
<td>4.69 ± 1.72</td>
<td>7.45 ± 2.56</td>
<td>37.05 ± 2.06</td>
</tr>
<tr>
<td></td>
<td>CD4</td>
<td>0.96 ± 0.59</td>
<td>1.67 ± 0.50**</td>
<td>56.62 ± 4.12</td>
</tr>
<tr>
<td></td>
<td>CD8</td>
<td>1.22 ± 0.46</td>
<td>1.66 ± 0.72</td>
<td>47.45 ± 14.18</td>
</tr>
<tr>
<td></td>
<td>B cell</td>
<td>3.20 ± 0.94</td>
<td>13.64 ± 5.63**</td>
<td>74.98 ± 7.18</td>
</tr>
<tr>
<td></td>
<td>Macrophage</td>
<td>7.70 ± 0.71</td>
<td>23.13 ± 8.08**</td>
<td>66.99 ± 8.42</td>
</tr>
<tr>
<td></td>
<td>PMN</td>
<td>0.56 ± 0.21</td>
<td>0.72 ± 0.26</td>
<td>23.34 ± 4.28</td>
</tr>
<tr>
<td></td>
<td>NK</td>
<td>3.94 ± 0.69</td>
<td>7.65 ± 1.70**</td>
<td>54.26 ± 11.37</td>
</tr>
<tr>
<td>8</td>
<td>CD3</td>
<td>5.94 ± 1.10</td>
<td>18.20 ± 2.13***</td>
<td>67.27 ± 5.05</td>
</tr>
<tr>
<td></td>
<td>CD4</td>
<td>1.29 ± 0.10</td>
<td>3.86 ± 0.45***</td>
<td>66.27 ± 4.56</td>
</tr>
<tr>
<td></td>
<td>CD8</td>
<td>2.20 ± 0.74</td>
<td>10.15 ± 1.25***</td>
<td>78.30 ± 6.69</td>
</tr>
<tr>
<td></td>
<td>B cell</td>
<td>2.44 ± 0.55</td>
<td>3.11 ± 0.20</td>
<td>21.82 ± 5.06</td>
</tr>
<tr>
<td></td>
<td>Macrophage</td>
<td>7.47 ± 0.49</td>
<td>10.59 ± 0.62***</td>
<td>29.14 ± 8.10</td>
</tr>
<tr>
<td></td>
<td>PMN</td>
<td>0.90 ± 0.11</td>
<td>1.96 ± 0.22***</td>
<td>53.19 ± 10.21</td>
</tr>
<tr>
<td></td>
<td>NK</td>
<td>4.40 ± 0.57</td>
<td>6.73 ± 0.43***</td>
<td>34.35 ± 10.22</td>
</tr>
</tbody>
</table>

$^a$ BALB/c mice were infected with 1 × 10$^6$ TCID$_{50}$ PFU of A2 or rA2-line19F RSV. Data are mean total numbers of BAL cells by subtype at day 5 p.i. per lung. Subtypes: lymphocyte gate, anti-CD3$^+$ (17A2) and anti-CD4$^+$ (GK1.5) CD4 T cells; lymphocyte gate, CD3$^+$ and anti-CD8$^+$ (53.6.7) CD8 T cells; CD3$^+$ and anti-CD45R/B220$^+$ (RA3-6B2) B cells; CD3$^-$ and anti-CD11b$^+$ (M1/70)$^+$ macrophages, dendritic cells, and monocytes; CD3$^-$ and anti-mouse Ly06G/Gr-1 (RB6-8C5)$^+$ polymorphonuclear cells (PMNs); CD3$^-$ and anti-mouse CD49b/integrin alpha 2 (DX5)$^+$ NK cells. **, $P < 0.01$, as determined by ANOVA; ***, $P < 0.001$, a significant increase in total BAL cell types in the rA2-line19F-infected group compared to the A2-infected group. Results are representative of two independent experiments for rA2-line19F challenge and mock challenge and one experiment for A2 challenge. The data are from no fewer than five mice per experiment.
F(ab'), 131-2G MAb treatment reduces pulmonary cell infiltrates and substance P levels. Prophylactic treatment with both the F(ab')2 and intact forms of 131-2G significantly decreased (P < 0.001) pulmonary inflammation, as indicated by the total BAL cell numbers (33% and 43% reduction, respectively) and BAL cell types (Fig. 4B and Table 2). With the F(ab')2 treatment, the most marked decrease in BAL cell types was for CD45R/B220 (RA3-6B2) cells (B cells; 81.63% ± 7.50%) and CD11b (M1/70) (macrophages, dendritic cells, and monocytes; 66.43% ± 10.30%) (Table 2).

To determine a potential role for substance P in rA2-line19F-associated disease, we tested and compared SP levels in the BAL specimens of rA2-line19F-, A2-, and mock-challenged mice (Table 3). The levels of SP in BAL specimens after mock challenge ranged from 238 to 278 pg/ml. SP levels after A2 challenge, as previously noted (13), were significantly increased. rA2-line19F challenge gave the highest pulmonary SP levels, and these levels were significantly above those for untreated rA2-line19F-challenged mice (Table 3).

F(ab'), 131-2G MAb inhibits rA2-line19F-induced airway dysfunction in BALB/c mice. Airway dysfunction, manifested by signs of increased airway resistance, is the hallmark feature of RSV bronchiolitis. The increase in breathing effort based on pulsus paradoxus is an indicator of airway dysfunction. As noted in Fig. 2, the increase in breathing effort with rA2-line19F-challenged mice peaked on day 8 p.i., and values for day 8 p.i. were used for this analysis. Prophylaxis with either form of MAb 131-2G markedly decreased (P ≤ 0.001) RSV rA2-line19F-induced breathing effort compared to that for untreated and nIgG antibody-treated mice (rA2-line 19F). The results for 131-2G F(ab')2 and rA2-line19F are representative of two independent experiments.
TABLE 2 Changes in the characteristics of the BAL cell infiltrate after prophylaxis with the intact anti-G protein MAb or F(ab’2) form of 131-2G

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Untreated</th>
<th>F(ab’2) 131-2G-treated</th>
<th>Intact 131-2G-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean no. of cells ± SE (10⁶)</td>
<td>Mean no. of cells ± SE (10⁶)</td>
<td>% reduction</td>
</tr>
<tr>
<td>CD3</td>
<td>6.46 ± 1.79</td>
<td>3.36 ± 0.27**</td>
<td>53.71 ± 19.69**</td>
</tr>
<tr>
<td>CD4</td>
<td>1.60 ± 0.46</td>
<td>0.65 ± 0.07**</td>
<td>63.71 ± 19.69**</td>
</tr>
<tr>
<td>CD8</td>
<td>1.66 ± 0.72</td>
<td>0.54 ± 0.17***</td>
<td>61.74 ± 19.69**</td>
</tr>
<tr>
<td>B cell</td>
<td>12.97 ± 5.16</td>
<td>2.18 ± 0.20***</td>
<td>81.74 ± 19.69**</td>
</tr>
<tr>
<td>Macrophage</td>
<td>23.36 ± 7.14</td>
<td>6.75 ± 3.90***</td>
<td>74.85 ± 19.69**</td>
</tr>
<tr>
<td>PMN</td>
<td>0.72 ± 0.23</td>
<td>0.44 ± 0.04**</td>
<td>44.78 ± 19.69**</td>
</tr>
<tr>
<td>NK</td>
<td>7.33 ± 1.64</td>
<td>3.76 ± 2.22**</td>
<td>65.38 ± 19.69**</td>
</tr>
</tbody>
</table>

Mice were treated with 300 μg of the intact or F(ab’2) form of 131-2G 2 days before RSV rA2-line19F (1 × 10⁶ TCID₅₀) challenge (n = 5 mice/group). Data are mean total numbers of BAL cells by subtype at day 5 p.i. per lung. Subtypes: lymphocytes gate, anti-CD3+ (17A2) and anti-CD4+ (GK1.5) CD4 T cells; lymphocyte gate, CD3+ and anti-CD8– (53-6.7) CD8 T cells; CD3+ and anti-CD45R/B220– (RA3-6B2) B cells; CD3+ and anti-CD11b– (M1/70) macrophages, dendritic cells, and monocytes; CD3+ and anti-mouse Ly6G/Gr-1 (RB6-8C5) polymorphonuclear cells (PMNs); CD3+ and anti-mouse CD49b/integrin alpha 2 (DX5)+ NK cells. The fold reduction is the difference in the number of treated mice relative to the number of untreated mice for the cell type. **, P ≤ 0.05 as determined by ANOVA; ***, P ≤ 0.001, a significant decrease in the number of untreated mice compared to the number receiving the indicated treatment. Results are representative of two independent experiments for rA2-line19F challenge and F(ab’2) 131-2G prophylaxis for rA2-line19F-challenged mice.

documented 8 days after challenge, and values for day 8 were used for these analyses. There was an increase in Muc5AC protein expression in the lungs of mice challenged with rA2-line19F but not in the lungs of A2-challenged mice (Fig. 5A), which was similar to the results of other studies (25). In a fashion similar to that for increased pulsus paradoxus, prophylactic treatment with both the F(ab’2) and intact forms of 131-2G significantly decreased (P = 0.001) Muc5AC levels compared to those in untreated and nlgG antibody-treated mice challenged with rA2-line19F (Fig. 5B). The ability of F(ab’2) 131-2G to so effectively decrease mucin production shows that this effect is also independent of decreasing virus replication.

Differences in pulmonary mucus production between A2 and rA2-line19F infections and between treatment with either form of MAb 131-2G and no treatment before rA2-line19F infection were evident in lung histopathology studies (Fig. 6A). Lung sections harvested 8 days p.i. showed PAS staining (a staining method used to detect intracellular pulmonary mucin levels)-stained cells for untreated mice. A2-challenged mice and MAb 131-2G-treated, rA2-line19F-challenged mice did not show PAS-stained cells. As expected, A2-challenged mice and rA2-line19F-challenged mice treated with the F(ab’2) form of 131-2G were positive for RSV antigens by immunofluorescent staining (Fig. 6B).

Immunohistochemical staining (IHC) for Muc5AC production analyzed using ImageJ software (see Materials and Methods) was used to quantify differences between treatment groups. Consistent with PAS-staining results, the percentages of Muc5AC-positive stained areas in the total epithelial area were 32.8% after rA2-line19F challenge, 32.2% after A2 challenge, 9.6% after mock challenge, and 10.8% and 11.6% for rA2-line19F-challenged mice given prophylaxis with intact and F(ab’2) forms of MAb 131-2G.

Cytokine responses. To address a potential mechanism for the decrease in pulsus paradoxus and mucin production after prophylaxis with the anti-RSV G protein MAb 131-2G, we determined the levels of pulmonary cytokines and chemokines in lung homogenates. As noted with earlier studies, elevated levels of Th2 cytokines, such as IL-4, IL-13, IL-5, and MCP-1, have been associated with the increased airway resistance, mucous production, and bronchoconstriction seen in patients with asthma. At 8 days p.i., significant increases (P ≤ 0.001) in the levels of IL-4, IL-5, IL-13, and MCP-1 were detected in untreated, nlgG antibody-treated, and untreated rA2-line19F-challenged mice (Fig. 7). These increases were significantly lower in rA2-line19F-challenged mice treated with either form of MAb 131-2G and in A2-challenged mice. In addition, substantially higher levels of IP-10 and IL-12 were detected in lung homogenates after prophylaxis with either form of the 131-2G MAb than in untreated or nlgG antibody-treated, rA2-line19F-challenged mice. A2-challenged mice also had higher levels of IL-12 and IP-10 (Fig. 7).

DISCUSSION

Development of an effective vaccine or treatment for RSV infection has not yet been achieved, though passive administration of an immune globulin with high titers of RSV antibodies or a neutralizing MAB is effective when given prophylactically. One possible explanation for success with antibody prophylaxis (4) and limited success with treatment of active infection could be that the virus-induced host inflammatory response is important to the dis-
ease process and is only partially responsive to stopping replication once infection is established (39, 40). Consequently, effective treatment of active infection may require both anti-inflammatory and antiviral components. RSV vaccines or prophylaxis may also be more effective if they have both anti-inflammatory and antiviral effects. There is increasing evidence for RSV G protein being an important contributor to RSV inflammation and disease pathogenesis, and thus it is a potential target for the anti-inflammatory component of vaccine and antiviral drug development strategies (22, 23, 41–44).

Since immune prophylaxis with palivizumab is about 50% effective in decreasing RSV hospitalizations (4), it is possible that binding G and blocking G-associated disease might improve the effectiveness of immune prophylaxis. The RSV G protein, G peptides, and secreted G have been associated with a number of immune and inflammatory responses. For example, vaccination with intact or secreted G and several peptides in or proximal to the central nonglycosylated region of G induce a Th2-biased memory response resulting in increased pulmonary inflammation and eosinophilia after RSV challenge (45–50). In other studies, the G protein has been associated with suppressing some immune responses, such as Toll-like receptor 3 (TLR3) or TLR4 induction of IFN-β (48), proinflammatory responses of lung epithelial cells (51), lymphoproliferation (52), and a number of other innate responses, including activation of dendritic cells (53, 54), enhanced cytotoxic T cell responses (55), and downregulation of type I IFN production by inducing suppression of cytokine signaling (SOCS) (56). The G protein has also been associated with depression of the respiratory rate (16), increased production of pulmonary substance P (15), and antibody decay activity (57).

Earlier studies showed that binding the G protein with MAb 131-2G prevented pulmonary inflammation after primary infection, pulmonary inflammation, and eosinophilia in FI-RSV-vaccinated mice and weight loss after primary infection or infection in FI-RSV-vaccinated mice (21–23). The availability of the rA2-line19F virus allowed us to demonstrate that MAb 131-2G prophylaxis also decreases breathing effort and pulmonary mucous production. These findings suggest additional clinical benefits of treatment or prophylaxis with this or a comparable anti-G protein MAb. These findings also expand the possible contributions of the G protein to the pathogenesis of RSV disease.

The potential importance of these findings to human RSV disease is highlighted by wheezing, often resulting in a diagnosis of bronchiolitis, being the hallmark clinical feature of RSV infection in children (32, 58). Wheezing is associated with increased airway resistance, which can result from bronchoconstriction or plugging of the airways with sloughed airway epithelial and inflammatory cells, other debris, and/or mucous. With rA2-line19F, we see increased pulsus paradoxus indicative of increased breathing effort, which indicates airway dysfunction. The data in this report and data from Moore et al. (25) are consistent with one or several mechanisms causing the increased breathing effort; i.e., infection with rA2-line19F is associated with the increased pulmonary inflammation, Th2 cytokine levels, and mucous production that parallel the increase in breathing effort. In addition, the data in this report show a decrease in breathing effort with F(ab')₂, MAb 131-2G treatment that correlates with decreases in BAL cell infiltrates (Fig. 5 and Table 2), pulmonary mucous production (Fig. 6), pulmonary Th2 cytokines (Fig. 7), and substance P levels (Table 3). The cellular infiltration and/or mucous production can plug airways, leading to increased breathing effort, and Th2 cyto-

![Figure 5](image-url) Effect of different virus strains and anti-RSV G protein MAb 131-2G treatment on mucin production in BALB/c mice. (A) BALB/c mice were challenged with mock-infected tissue culture supernatant (mock) or 1 × 10⁶ TCID₅₀ of rA2-line19F or A2 RSV (n = 5 mice/group). Lungs were harvested 8 days p.i., and lung homogenates were tested for Muc5AC levels by ELISA. Data are means ± SEMs. ***, significant difference (P ≤ 0.001; ANOVA) between values for rA2-line19F-challenged mice and other indicated groups. (B) BALB/c mice were treated 2 days before rA2-line19F challenge with the intact or F(ab')₂ form of either 131-2G or control antibody (nIgG) (n = 5 mice/group). Lungs were harvested 8 days p.i., and lung homogenates were tested for Muc5AC levels. Data are means ± SEMs. ***, significant difference (P ≤ 0.001; ANOVA) between values for untreated rA2-line19F-challenged mice and other indicated groups. Results are representative of two independent experiments for untreated and F(ab')₂ 131-2G-treated mice challenged with rA2-line19F. Data for challenge with A2 and for prophylaxis with intact 131-2G are from one experiment.
cytokines IL-13, IL-4, and IL-5, and the chemokine MCP-1. Prophylaxis with MAb 131-2G decreased both mucous production and the expression of these cytokines and the chemokine. As noted with earlier studies, elevated levels of Th2 cytokines, such as IL-4 and IL-13, have been associated with the increased airway resistance, mucous production, and bronchoconstriction seen in patients with asthma (63–67). Blocking IL-13 and IL-4 has been shown to significantly inhibit mucus overproduction in a murine model, and local administration of recombinant IL-13 to nonimmunized mice induces goblet cell hyperplasia (43). The Th2 cytokine IL-5 is associated with allergic asthmatic responses in the lung (68). MCP-1 was also elevated and regulates migration and infiltration of monocytes/macrophages, which could indirectly contribute to mucous secretion (69) and airway dysfunction (70).

The increase in IL-12 and IP-10 with F(ab’)2, MAb 131-2G prophylaxis is consistent with a shift toward a Th1 response from a Th2 response with the decrease in lung disease and suggests that the virus is actively suppressing the production of these mediators. IL-12 has been associated with a pathophysiologically milder response to the infection (71). Production of IL-12 is thought to favor differentiation and function of (Th1) T cells while inhibiting the differentiation of Th2 cells. In many viral infections, IL-12 promotes viral clearance and host recovery from infection (71–74).

We chose to test for pulmonary substance P levels because the G protein has been associated with elevated levels in BAL fluid and substance P has been linked to increased inflammation and airway resistance (15). Our finding that F(ab’)2, MAb 131-2G prophylaxis decreased pulmonary substance P levels is consistent with previous work linking G to induction of substance P in the lung, as noted above, as well as a study suggesting that substance P participates in G downregulation of the respiratory rate in mice (16). Substance P has been suggested as an important contributor to bronchospasm, mucus secretion, and inflammation in asthma and various infections (75).

We hypothesize that some of the disease-modifying effects of MAb 131-2G noted in this study are linked to G binding to CX3CR1 through its CX3C chemokine motif (18). Several studies using a virus with a Cys-to-Arg mutation at amino acid position 186 in the CX3C motif (CX3R) and anti-CX3CR1 antibodies suggest a link between G binding to CX3CR1 and RSV disease. In one study, FI-RSV-vaccinated mice challenged with a virus that lacked G or the CX3R virus or were given prophylactic treatment with anti-CX3CR1 antibody before challenge had a marked decrease in the enhanced pulmonary inflammation otherwise seen in FI-RSV-vaccinated mice (14). In another study, mice challenged with the CX3R virus had significantly more effector T cells trafficking into the lungs and a higher percentage of T cells producing IFN-γ than mice challenged with the wild-type virus (17). In a third study, mice given the RSV G protein intravenously had a decrease in respiratory rate but mice given the CX3R G protein or mice treated with anti-CX3CR1 antibody before G was given did not show decreased respiratory rates (16). In this study, binding substance P with antibody also eliminated G’s reduction in respiratory rates, raising the possibility that the effect of the G protein, CX3CR1, and substance P on RSV disease may be linked. Interestingly, a recent study noted that fractalkine and CX3CR1 contribute to inflammation and a Th2-biased response in a mouse model of allergic inflammation (76). We have initiated studies to determine if, in fact, the CX3C motif in G and G binding to kines and substance P have been associated with bronchoconstriction, which would also lead to increased breathing effort.

Airway mucus hypersecretion is an important feature of chronic airway inflammatory diseases and has been detected in some autopsy specimens from children who died with severe RSV disease (59–61). Goblet cell hyperplasia plays a crucial role in mucus hypersecretion, and Muc5AC is a marker for increased goblet cell mucin production during airway inflammation (62). Hashimoto et al. demonstrated that RSV infection augmented Muc5AC and Gob-5 gene expression and staining in the ovalbumin allergy model of lung inflammation in mice (43). In the present study, RA2-line19F induced mucous production, the Th2-type cytokines IL-4, and IL-5, and the chemokine MCP-1. Prophylaxis with MAb 131-2G decreased both mucous production and the expression of these cytokines and the chemokine. As noted with earlier studies, elevated levels of Th2 cytokines, such as IL-4 and IL-13, have been associated with the increased airway resistance, mucous production, and bronchoconstriction seen in patients with asthma (63–67). Blocking IL-13 and IL-4 has been shown to significantly inhibit mucus overproduction in a murine model, and local administration of recombinant IL-13 to nonimmunized mice induces goblet cell hyperplasia (43). The Th2 cytokine IL-5 is associated with allergic asthmatic responses in the lung (68). MCP-1 was also elevated and regulates migration and infiltration of monocytes/macrophages, which could indirectly contribute to mucous secretion (69) and airway dysfunction (70).

The increase in IL-12 and IP-10 with F(ab’)2, MAb 131-2G prophylaxis is consistent with a shift toward a Th1 response from a Th2 response with the decrease in lung disease and suggests that the virus is actively suppressing the production of these mediators. IL-12 has been associated with a pathophysiologically milder response to the infection (71). Production of IL-12 is thought to favor differentiation and function of (Th1) T cells while inhibiting the differentiation of Th2 cells. In many viral infections, IL-12 promotes viral clearance and host recovery from infection (71–74).

We chose to test for pulmonary substance P levels because the G protein has been associated with elevated levels in BAL fluid and substance P has been linked to increased inflammation and airway resistance (15). Our finding that F(ab’)2, MAb 131-2G prophylaxis decreased pulmonary substance P levels is consistent with previous work linking G to induction of substance P in the lung, as noted above, as well as a study suggesting that substance P participates in G downregulation of the respiratory rate in mice (16). Substance P has been suggested as an important contributor to bronchospasm, mucus secretion, and inflammation in asthma and various infections (75).

We hypothesize that some of the disease-modifying effects of MAb 131-2G noted in this study are linked to G binding to CX3CR1 through its CX3C chemokine motif (18). Several studies using a virus with a Cys-to-Arg mutation at amino acid position 186 in the CX3C motif (CX3R) and anti-CX3CR1 antibodies suggest a link between G binding to CX3CR1 and RSV disease. In one study, FI-RSV-vaccinated mice challenged with a virus that lacked G or the CX3R virus or were given prophylactic treatment with anti-CX3CR1 antibody before challenge had a marked decrease in the enhanced pulmonary inflammation otherwise seen in FI-RSV-vaccinated mice (14). In another study, mice challenged with the CX3R virus had significantly more effector T cells trafficking into the lungs and a higher percentage of T cells producing IFN-γ than mice challenged with the wild-type virus (17). In a third study, mice given the RSV G protein intravenously had a decrease in respiratory rate but mice given the CX3R G protein or mice treated with anti-CX3CR1 antibody before G was given did not show decreased respiratory rates (16). In this study, binding substance P with antibody also eliminated G’s reduction in respiratory rates, raising the possibility that the effect of the G protein, CX3CR1, and substance P on RSV disease may be linked. Interestingly, a recent study noted that fractalkine and CX3CR1 contribute to inflammation and a Th2-biased response in a mouse model of allergic inflammation (76). We have initiated studies to determine if, in fact, the CX3C motif in G and G binding to

FIG 6 Mucin production and RSV replication associated with RSV rA2-line19F challenge and anti-G protein MAb 131-2G prophylaxis. BALB/c mice were challenged with mock-infected tissue culture supernatant (mock) or 1 × 106 TCID50 of A2 or rA2-line19F and were untreated or treated 2 days before challenge with the intact or F(ab’)2 form of both intact 131-2G and control immune globulin (nIgG) (n = 5 mice/group). Lungs were harvested 5 days p.i. for RSV antigen immunofluorescence and 8 days p.i. for PAS staining. (A) Representative photomicrographs of PAS-stained lung tissue collected at 8 days p.i. Reddish-purple color (e.g., evident in panels labeled A2 and rA2-line19F; n = 3 mice/group). Results are representative of two independent experiments for untreated or 131-2G F(ab’)2-treated mice challenged with rA2-line19F.

October 2013 Volume 87 Number 20 jvi.asm.org 10963
CX3CR1 play a role in the manifestations of RSV disease noted in the present study.

Importantly, as noted in earlier studies, results with the F(ab')₂ form of the MAb show that the impact of G on disease is independent of virus replication. Binding G with the F(ab')₂ form of 131-2G does not decrease virus replication, which is consistent with the previous findings with this MAb (22) and another non-neutralizing anti-G protein MAb, IC2 (77). One chimera of Mab, IC2 with an intact γ chain, prevented virus replication, while another chimera with a glycosyl mutation that did not activate complement or binding to the Fc receptor was less effective in preventing virus replication. These findings are consistent with virus
neutralization by antibody-dependent cell-mediated cytotoxicity (ADCC)-dependent or complement-mediated mechanisms. In fact 131-2G, as noted in the present study and previous studies (22, 23), effectively neutralizes RSV in vivo and thus in the mouse impacts RSV disease both by downregulating G-induced inflammation and immune modulation and by decreasing virus replication. If it impacts both in humans, it might prove more effective in decreasing disease than antiviral treatment that only decreases virus replication.

The ability of an anti-G protein MAb to so effectively decrease pulmonary mucous production and breathing effort is somewhat surprising, since the difference between RSV A2 and rA2-line19F is five amino acid changes in the F protein and no differences in the G protein. Consequently, both proteins must be involved in rA2-line19F infection-associated mucous production and airway reactivity. The G and F proteins do interact during infection, and this may explain a joint role in these manifestations of infection (78). Alternatively, the role of each may have independent effects that in concert result in mucous overproduction and increased breathing effort. We are beginning studies to better understand the role of F and G in these manifestations of RSV disease.

Two limitations need to be considered in interpreting the data presented in the report. First, pulsus paradoxus, or breathing effort, is an indication of airway dysfunction but does not indicate the location of this dysfunction. Previous work, however, has shown that increases in pulsus paradoxus, or breathing effort, in the mouse do correspond to increases in airway hyperresponsiveness (AHR) in mice challenged with RSV rA2-line19F. AHR was measured using a small animal ventilator and methacholine challenge (38), and the correspondence between this measurement and increased breathing effort supports that breathing effort in this model indicates dysfunction in the lung. We chose to use breathing effort because it is less invasive than measuring AHR and it allows serial measures in the same mouse. Second, it is important to recognize that findings in mice may, or may not, apply to humans. Thus, although these data suggest a substantial role for the G protein in the pathogenesis of human RSV disease, this suggestion needs to be confirmed by studies with humans.

Together, the data presented in this report identify additional roles for the RSV G protein in disease pathogenesis, i.e., mucous production and airway dysfunction. These results and the results of other studies of G suggest that understanding G’s role in the pathogenesis of disease is likely essential to understanding RSV disease and likely important in the approach to its treatment and prevention. Finally, if G’s impact on human RSV disease is similar to its impact on disease in mice, binding G with a MAB or antiviral drug with activities similar to those of 131-2G MAb could improve the effectiveness of RSV prophylaxis and/or treatment. The data also suggest that inducing antibodies with activity similar to that of MAB 131-2G might improve a vaccine’s ability to prevent disease with later RSV infection.

ACKNOWLEDGMENTS

This work was supported by NIH grant 1U19AI095227 awarded to M.L.M. and L.J.A., funding from Children’s Healthcare of Atlanta, support from the Immunology Core of Emory’s Pediatric Research Center, and Emory Vaccinology Training Grant (VTP) T32 5T32AI074492-03. Martin L. Moore was supported by NIH 1R01AI087798 and NIH 1U19AI095227.

We thank Carla J. Shoffeitt for histology slide preparations and Sica L. Gabriel for histology slide analysis.

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

REFERENCES


41. Oshinsky CM, Krunskosky TM, Barber J, Jones LP, Tripp RA. 2009. Respiratory syncytial virus proteins modulate suppressors of cytokine sig-


