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

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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

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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.
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 rA2-line19F provides additional clinically relevant outcomes to study in mice (25, 33). In this study, we chose to determine if the F(\(\text{ab}'\))\(_2\) 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(\(\text{ab}'\))\(_2\) form of 131-2G to eliminate decreased virus replication as a reason for the observed effects. Our results show that prophylaxis with the F(\(\text{ab}'\))\(_2\) 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 tribromoethanol, 180 to 250 mg/kg in tertiary amyl alcohol) and chilled water and food ad libitum. In study 2, mice were intraperitoneally treated with 300 mg free minimal essential medium (MEM) (volume, 50 \(\mu\)l) with no treatment. Mice were inoculated onto subconfluent HEP-2 cells in 96-well microtiter plates. After 2 h of adsorption at 37°C, 180 \(\mu\)l of tissue culture media was added, and 5 days later, the cells were fixed with 80% acetone in 1X 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 1X 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 1X 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, St. Louis, MO) 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(\(\text{ab}'\))\(_2\) fragment preparation and treatment. Monoclonal antibody 131-2G F(\(\text{ab}'\))\(_2\) 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(\(\text{ab}'\))\(_2\) fragments were dialyzed and concentrated using a Centricon spin column (Millipore, Temecula, CA) with a 30-kDa cutoff. The purity of the F(\(\text{ab}'\))\(_2\) 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-
lus amebocyte lyase 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 (gene) (forward primer, 5′-GGC AAA TAT GGA AAC ATA GCT GAA-3′; reverse primer, 5′-TCT TTT TCT AGG ACA TTT TAY TGA ACA G-3′; probe, 5′-6-carboxyfluorescin (FAM)-TCT CCG TCT TCT AGG CCC TCG TC-3′) were designed by using Primer Express software (PE Applied Biosystems, Foster City, CA). Amplification efficiencies were measured for each sample to the standard curve generated and expressed as a percent sample bound/maximum bound versus the standard SP concentration. The intra- and interassay coefficients of variation were 5% and 11%, respectively.

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) using procedures described by Tripp et al. (12). Briefly, BALF 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 washed 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) (polymorphonuclear cells [PMNs]), anti-mouse CD49b/integrin alpha 2 (DX5) (NK and NT cells), and mouse isotype antibody controls (all from eBiosciences, San Diego, CA) diluted in staining buffer. The antibodies and patterns of cell surface markers were determined for 10,000 lymphocyte-gated events analyzed on a BD LSRII flow cytometer (BD Biosciences, Mountain View, CA), and data were analyzed using FlowJo software (TreeStar, Ashland, OR).
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 (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 25 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 × 10⁶ TCID₅₀) 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 TCID₅₀ 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
TABLE 1 Pulmonary leukocyte trafficking after rA2-line19F and A2 RSV infectionsa

<table>
<thead>
<tr>
<th>Day postinfection</th>
<th>Phenotype</th>
<th>Mean total no. of A2 cells ± SE (10⁵)</th>
<th>Mean total no. of rA2-line19F cells ± SE (10⁵)</th>
<th>% increase, A2 to rA2-line19F</th>
</tr>
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<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>
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<tr>
<td></td>
<td>CD4</td>
<td>0.22 ± 0.05</td>
<td>0.35 ± 0.09</td>
<td>33.77 ± 16.89</td>
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<tr>
<td></td>
<td>CD8</td>
<td>0.24 ± 0.02</td>
<td>0.49 ± 0.08***</td>
<td>49.53 ± 5.65</td>
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<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>
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<tr>
<td></td>
<td>NK</td>
<td>1.86 ± 0.75</td>
<td>3.71 ± 1.14**</td>
<td>49.86 ± 9.30</td>
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<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>
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<tr>
<td></td>
<td>CD4</td>
<td>0.20 ± 0.14</td>
<td>1.36 ± 0.35***</td>
<td>83.81 ± 13.45</td>
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<td>CD8</td>
<td>0.16 ± 0.06</td>
<td>0.94 ± 0.09***</td>
<td>83.05 ± 6.07</td>
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<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>
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<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>
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<td>0.66 ± 0.12</td>
<td>41.07 ± 10.01</td>
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<td></td>
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<td>2.04 ± 0.70</td>
<td>4.71 ± 0.22**</td>
<td>56.77 ± 19.98</td>
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<td>5</td>
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<td>4.69 ± 1.72</td>
<td>7.45 ± 2.56</td>
<td>37.05 ± 2.06</td>
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<tr>
<td></td>
<td>CD4</td>
<td>0.96 ± 0.59</td>
<td>1.67 ± 0.50**</td>
<td>56.62 ± 11.20</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>
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<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>
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<tr>
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<td>Macrophage</td>
<td>7.70 ± 0.71</td>
<td>23.13 ± 8.08**</td>
<td>66.99 ± 8.42</td>
</tr>
<tr>
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<td>PMN</td>
<td>0.56 ± 0.21</td>
<td>0.72 ± 0.26</td>
<td>23.34 ± 4.28</td>
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<tr>
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<td>NK</td>
<td>3.94 ± 0.69</td>
<td>7.65 ± 1.70**</td>
<td>54.26 ± 11.37</td>
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<td>8</td>
<td>CD3</td>
<td>5.94 ± 1.10</td>
<td>18.20 ± 2.13**</td>
<td>67.27 ± 5.05</td>
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<td></td>
<td>CD4</td>
<td>1.29 ± 0.10</td>
<td>3.86 ± 0.45***</td>
<td>66.27 ± 4.56</td>
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<tr>
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<td>CD8</td>
<td>2.20 ± 0.74</td>
<td>10.15 ± 1.25**</td>
<td>78.30 ± 6.69</td>
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<td>3.11 ± 0.20</td>
<td>21.82 ± 5.06</td>
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<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>

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 66.999% ± 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⁺ CD53.67⁺ cells (T cells), and a 56.62% ± 11.20% increase in CD4⁺ (Gr1.k)⁺ cells. By contrast, day 8 p.i., the most prominent increase in a BAL cell type for rA2-line19F-infected mice compared to that for A2-infected mice was the 78.30% ± 6.59% increase in CD8⁺ CD53.67⁺ cells (T cells). Pulsum 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')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.

F(ab')₂, 131-2G MAb treatment protects mice from RSV-induced weight loss but not pulmonary virus replication. Treatment with the F(ab')₂ and intact forms of 131-2G prevented much of the weight loss seen in mice challenged with rA2-line19F (P = 0.001) (Fig. 3). As expected, the intact form of anti-RSV G MAb 131-2G decreased pulmonary virus load (on day 5 p.i.; P = 0.001), while the F(ab')₂ form had no effect on virus load (Fig. 4A). The RSV real-time PCR results for BAL specimens (5 mice/group) on day 5 p.i. also showed that 131-2G F(ab')₂ prophylaxis did not affect virus replication; i.e., the C₅ values were 34 ± 2 for untreated, nlgG antibody-treated, or 131-2G F(ab')₂-treated mice challenged with RSV (data not shown). Thus, the effects on disease of 131-2G F(ab')₂ prophylaxis are not the result of a decrease in virus replication but are presumably from binding to G and blocking G-induced host responses to infection.
F(ab’), 131-2G MAb treatment reduces pulmonary cell infiltrates and substance P levels. Prophylactic treatment with both the F(ab’), 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’), treatment, the most marked decrease in BAL cell types was for CD45R/B220 (RA3-6B2) (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 A2 on day 5 p.i. (Table 3). Prophylaxis with F(ab’), 131-2G, as well as with intact 131-2G, significantly decreased pulmonary SP (42.52% ± 4.09% and 43.79% ± 6.13%, respectively, compared to that for untreated rA2-line19F-challenged mice) at day 5 p.i., to levels below that for A2-challenged mice (Table 3).

F(ab’), MAb 131-2G 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 challenged with rA2-line19F (Fig. 4C). The values for MAb 131-2G-treated mice were similar to those for mock-challenged mice (rA2-line 19F). The results for 131-2G F(ab’), and rA2-line19F are representative of two independent experiments.
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 mucous 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 (a staining method used to detect intracellular pulmonary mucin levels)-stained cells for untreated, A2-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).

**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-

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### 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 Mean no. of cells ± SE (10^6)</th>
<th>F(ab')2 131-2G-treated Mean no. of cells ± SE (10^6)</th>
<th>% reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>6.46 ± 1.79</td>
<td>3.26 ± 0.51**</td>
<td>52.25**</td>
</tr>
<tr>
<td>CD4</td>
<td>1.60 ± 0.46</td>
<td>0.65 ± 0.07**</td>
<td>61.38**</td>
</tr>
<tr>
<td>CD8</td>
<td>1.66 ± 0.72</td>
<td>0.54 ± 0.04**</td>
<td>68.35**</td>
</tr>
<tr>
<td>B cell</td>
<td>12.97 ± 5.16</td>
<td>2.18 ± 0.20**</td>
<td>82.63**</td>
</tr>
<tr>
<td>Macrophage</td>
<td>23.36 ± 7.14</td>
<td>6.75 ± 3.90**</td>
<td>71.51**</td>
</tr>
<tr>
<td>PMN</td>
<td>0.72 ± 0.23</td>
<td>0.44 ± 0.04**</td>
<td>54.85**</td>
</tr>
<tr>
<td>NK</td>
<td>7.33 ± 1.64</td>
<td>3.76 ± 2.22**</td>
<td>50.74**</td>
</tr>
</tbody>
</table>

**a** Mice were treated with 300 µg of the intact or F(ab')2 form of 131-2G 2 days before RSV rA2-line19F (1 × 10^6 TCID_{50}) challenge (n = 5 mice/group). 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 Ly6G/Gr-1 (RB6-8C5) polymorphonuclear cells (PMNs); CD3+ and anti-mouse CD49b/integrin alpha 2 (DX5)- NK cells. The fold reduction is the decrease 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 of rA2-line19F-challenged mice.

### TABLE 3 Differences between SP levels in BAL cells of A2- and rA2-line19F-challenged mice and between untreated and MAB 131-2G-treated mice challenged with rA2-line19F

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Substance P level in BAL cells (pg/ml ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2</td>
<td>432.58 ± 54.34</td>
</tr>
<tr>
<td>rA2-line19F**</td>
<td>620.91 ± 30.30</td>
</tr>
<tr>
<td>rA2-line19F+ F(ab')2 131-2G***</td>
<td>355.85 ± 10.95</td>
</tr>
<tr>
<td>rA2-line19F+ intact 131-2G**</td>
<td>349.92 ± 47.37</td>
</tr>
<tr>
<td>Mock</td>
<td>258.21 ± 20.86</td>
</tr>
<tr>
<td>rA2-line19F+ F(ab')2 nlgG</td>
<td>628.36 ± 17.31</td>
</tr>
<tr>
<td>rA2-line19F+ intact nlgG</td>
<td>617.52 ± 8.97</td>
</tr>
</tbody>
</table>

**a** BALGc mice were infected with 1 × 10^6 TCID_{50} of rA2-line19F or A2 RSV. Mice were treated with 300 µg of the intact or F(ab')2 form of 131-2G or control immunoglobulin (nlgG) 2 days before RSV rA2-line19F challenge (n = 5 mice/group). Data are means ± SEMs. BAL samples were collected on day 5 p.i. **, P ≤ 0.05 as determined by ANOVA), a significant difference between the levels of substance P in rA2-line19F-challenged and A2-challenged mice; ***, P ≤ 0.001 (determined by ANOVA), a significant difference between the levels of substance P in untreated mice and rA2-line19F-challenged mice receiving indicated treatment. Results are representative of two independent experiments for untreated and F(ab')2 131-2G-treated rA2-line19F-challenged mice. The data for intact-131-2G-treated mice and A2-challenged mice are from one experiment. 

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*Effects of Anti-G Protein F(ab')2 MAb*
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-

![FIG 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 x 10^6 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.
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 × 10^6 TCID_{50} 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 rA2-line19F-labeled panel) indicates PAS-positive cells indicative of mucin (n = 3 mice/group). (B) Representative photomicrographs of immunofluorescence staining for RSV from the groups of mice noted above at day 5 p.i. Green indicates RSV proteins in the cells (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.

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-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
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

**FIG 7** Effect of anti-G protein MAb 131-2G prophylaxis on pulmonary cytokine levels in A2-line19F RSV-challenged mice. BALB/c mice were challenged with mock-infected tissue culture supernatant (mock) or 1 × 10^6 TCID_50 of rA2-line19F RSV with or without treatment (2 days before challenge) with the intact or F(ab’)_2 form of 131-2G and normal immune globulin (nIgG). Lungs were harvested 8 days p.i., and lung homogenates were tested by Luminex multiplex assay for IL-13 (A), IL-5 (B), IL-4 (C), MCP-1 (D), IP-10 (E), or IL-12 (F). All values are expressed as pg/mg of protein. Data are means ± SEMs. ***, significant difference (P ≤ 0.001; ANOVA) between values for untreated and treated (as indicated) challenged mice. Results are representative of two independent experiments for untreated and 131-2G F(ab’)_2-treated mice. Data for challenge with A2 and for prophylaxis with intact 131-2G are from one experiment.
neutralization by antibody-dependent cell-mediated cytotoxicity (ADCC)-dependent or complement-mediated mechanisms. In- tect 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 vi- rus 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, pulsum paradoxus, or breathing ef- fort, is an indication of airway dysfunction but does not indicate the location of this dysfunction. Previous work, however, has shown that increases in pulsum paradoxus, or breathing effort, in the mouse do correspond to increases in airway hyperresponsive- ness (AHR) in mice challenged with RSV rA2-line19F. AHR was measured using a small animal ventilator and methacholine chal- lenge (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 in disease in mice, binding G with a MAB or antiviral drug with activities similar to those of 131-2G MAB could im- prove 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.

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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


