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Potent High-Affinity Antibodies for Treatment and Prophylaxis of Respiratory Syncytial Virus Derived from B Cells of Infected Patients

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Native human Abs represent attractive drug candidates; however, the low frequency of B cells expressing high-quality Abs has posed a barrier to discovery. Using a novel single-cell phenotyping technology, we have overcome this barrier to discover human Abs targeting the conserved but poorly immunogenic central motif of respiratory syncytial virus (RSV) G protein. For the entire cohort of 24 subjects with recent RSV infection, B cells producing Abs meeting these stringent specificity criteria were rare, <10 per million. Several of the newly cloned Abs bind to the RSV G protein central conserved motif with very high affinity (Kd 1–24 pM). Two of the Abs were characterized in detail and compared with palivizumab, a humanized mAb against the RSV F protein. Relative to palivizumab, the anti-G Abs showed improved viral neutralization potency in vitro and enhanced reduction of infectious virus in a prophylaxis mouse model. Furthermore, in a mouse model for postinfection treatment, both anti-G Abs were significantly more effective than palivizumab at reducing viral load. The combination of activity in mouse models for both prophylaxis and treatment makes these high-affinity human-derived Abs promising candidates for human clinical testing. The Journal of Immunology, 2009, 183: 0000 – 0000.

Respiratory syncytial virus (RSV), as a leading cause of pneumonia and bronchiolitis in infants and young children worldwide, is a high-priority target for development of an effective treatment or preventive therapy. Hospitalization rates for children younger than 5 years old are 3 times higher than for influenza and parainfluenza (1). RSV also causes severe disease in the elderly and those of any age with compromised cardiac, pulmonary, or immune systems (2–4). In the United States, it is estimated that >100,000 RSV-infected young children and elderly are hospitalized annually for supportive care (1, 2). The first candidate vaccine, formalin-inactivated RSV, was associated with enhanced disease when vaccinated children were subsequently naturally infected with RSV (5). No RSV subunit or live virus vaccine tested to date in humans has been shown to be effective. Accordingly, the only widely used intervention at present is palivizumab (Synagis), a humanized murine mAb against the RSV F protein licensed for RSV prophylaxis in high-risk infants and young children (6). A successor, motavizumab, with the same specificity but engineered to have higher affinity, 35 pM vs 2.6 nM (7), is currently in clinical trials. Although palivizumab has a role in decreasing hospitalizations, a significant number of treated infants remain susceptible to the disease. The hospitalization rate for premature infants treated with palivizumab was reported to be 5.8% vs 11% for untreated controls (6), implying ~50% breakthrough disease despite prophylactic therapy. Palivizumab has not been effective for postinfection therapy (8). The only approved postinfection therapeutic, ribavirin, is not highly effective and is not commonly used (9). In short, >50 years since the discovery of RSV, therapeutic options for this major medical problem remain highly unsatisfactory.

There are two major immunogenic proteins on the surface of the virus: the F (fusion) and G (attachment factor) proteins (10). The F protein is highly conserved and is required for infect cells. In contrast, the G protein is not required to infect cells and is highly variable; indeed, the two broad subtypes of RSV, A and B, are defined by differences in the G protein (11). Based on these characteristics, the F protein was considered an attractive target for mAb therapy as a successor to polyclonal RSV-neutralizing serum therapy (Respigam). Despite high variability in the G protein ectodomain (12), there are two conserved regions: an alternative initiation site that results in a secreted soluble form of the protein (13); and a central cysteine-noose region (14, 15). RSV actively subverts the host immune response (16–18), and the G protein has drawn increasing attention for its role in enhancing RSV disease (16, 19–26). For example, mice infected with RSV lacking the G protein alternative initiation site, and thus the capacity to express soluble G protein, had a milder disease course: 10-fold lower viral load at day 4 postinfection (near the peak for viral load); and a minimal increase in lung-infiltrating inflammatory cells at day 7 (the time when inflammation peaks with wild-type virus infection; Ref. 22). Several mechanisms are likely responsible for these effects (17), including a role for the G protein in suppressing TLR4 signaling, an effect mapped to the central conserved cysteine-noose region of the G protein (23, 24). Another potential mechanism for the immune modifying effects of the G protein is its...
interaction with the fractalkine receptor, CX3CR1, a key mediator of cell trafficking to the infected lung (15, 21). Consistent with these findings, human polymorphisms showing reduced activity of either TLR4 (27) or CX3CR1 (28) were significantly overrepresented in studies of infants with severe RSV disease.

Accumulating evidence suggests that Abs to the RSV G protein might be useful candidates for RSV therapy. Murine Abs to the central conserved region of the G protein have shown efficacy in prophylactic mouse (29) and cotton rat models (30). A recent report showed that such a murine mAb delivered postinfection decreased both viral replication and the associated pulmonary inflammatory response (31). Furthermore, the antiviral and immunomodulatory features of the mAb were shown to be separable functions by comparing the full IgG to an F(ab')2 fragment (32).

Fully human mAbs are increasingly recognized as superior therapeutic candidates over mouse-derived products (33, 34), because the mAbs have been naturally selected and therefore have high affinity, are stable, and lack off-target reactivity against human Ags. Compared with hyperimmunized mice, however, the frequency of favorable clones in the human repertoire is typically too low for practical recovery. In this report, we show successful application of a novel single cell phenotyping technology (35) to identify and recover extremely rare human B cells expressing high-affinity Abs to the central conserved cysteine-noose region of the RSV G protein. Efficacy of these Abs for viral clearance is described in mouse models for both prophylactic and postinfection treatment of RSV infection.

Materials and Methods

Memory B cell isolation, EBV transformation, and culture

Blood was collected from patients 1–4 mo after a documented RSV infection (by nasal swab RT-PCR or by an Ag ELISA assay). All samples were collected under informed consent either at the University of Rochester Medical Center or through Novatech Australia. PBMCs were isolated by standard Histopaque density gradient separation (Sigma-Aldrich), frozen, and shipped in a dry nitrogen container (Princeton CryoTech). PBMCs were thawed, diluted in 10 ml of IMDM (Mediatech), and centrifuged through 1 ml of heat-inactivated FBS (Invitrogen) to remove cellular debris. B cells were enriched using the Miltenyi B cell isolation kit II (Miltenyi Biotec) and then transformed as previously described (33). Titered, direct-pelleted EBV (Advanced Biotechnologies) was added to isolated B cells at 1/100 final dilution for 2 h at 37°C; then the cells were washed three times and plated.

To survey the Ab repertoire of a sample, B cells were plated at 10^6 cells/ml in 96-well round-bottom plates in IMDM, 20% heat-inactivated FBS, 20% giant cell tumor-conditioned medium (BioVeris), 2 μg/ml CpG (Invivogen), and 10 ng/ml IL-10 (R&D Systems), supplementing with an equal volume of fresh medium every 2–3 days and expanding the cells to additional wells to maintain cell density. After 6–7 days in culture, cells were collected, washed with medium, and assayed by single-cell phenotyping using 500 B cells per well.

For recovery of favorable B cells, a larger aliquot of B cells was transformed and then further enriched using goat anti-human IgG microbeads (Miltenyi Biotec). The IgG+ cells were plated at 300 cells (60 μl) per well in 96-well round-bottom tissue-culture-treated plates, which had been seeded 1 day earlier with irradiated (500 R) feeder embryonic fibroblasts (American Type Culture Collection; ATCC). On day 3, 30 μl of medium were added. After 6 days in culture, the cells were resuspended, and one-half of the cells transferred to a new microplate, washed three times with medium, and assayed by single-cell phenotyping. Because the cells in the master plate experienced several rounds of replication, the positive assay wells typically had 2–10 replicate spots. Recovery of cells of interest from the positive wells was accomplished by replating the corresponding master plate well at limiting dilution using the same feeder cells and medium. After 4–6 days in culture, supernatants were collected from these wells for confirmatory RSV Ga-specific ELISA.

CellSpot single-cell phenotyping and ELISA assays

RSV G protein from strain A2 (Ga), RSV G protein from strain B1 (Gb), and RSV F proteins were purified from virus-infected cells as previously described (15). These native proteins (50 μg) were conjugated to customized dyed 280-nm-diameter latex beads (35) in 50 mM borate buffer (pH 9.5) overnight, followed by addition of 3 mg/ml sodium cyanoborohydride at 25°C for 2 h. BSA (Sigma-Aldrich) solution was added to the suspension to a final concentration of 1%; the beads were washed in the same buffer and stored in 50 mM borate/1% BSA, 0.05% Tween 20 at 4°C.

Microplates (96-well μClear high-bind black; Greiner North America) were carried 5 μl of the first amplification reaction was used. These primers carried 5'-BglII and 3'-XbaI restriction sites. Thirty PCR cycles were performed per round with identical conditions for the first and second rounds of amplification. For each reaction, 5 μl were separated on a 1% agarose gel and stained with ethidium bromide. The V-C PCR product was predicted to amplify rearranged fragments of 500 and 450 bp. PCR bands with a molecular size of ~500 bp indicated a positive result. PCR products were purified (Qiagen gel purification kit), and extracted PCR products were sequenced using specific constant region primers. Sequences were analyzed by VBASE2 to identify germline V(D)J, Ig CDR3 length, and somatic hypermutations.

Each PCR fragment was digested and cloned into an Ig expression vector carrying the constant region of human V1, Cε or Cα, respectively, for in vitro Ab production in a mammalian expression system. Each Ab was transiently produced in HEK293 cells and purified by chromatography on immobilized protein A (Aragen Bioscience).

Epitope mapping

For high-resolution epitope mapping, an array of 12-mer Ga-derived peptides, each shifted by one residue (JPT Peptide Tech), was probed with the Abs at 1 μg/ml and detected using peroxidase-labeled goat anti-human IgG (Jackson ImmunoResearch) in combination with SuperSignal chemiluminescence detection (Pierce). Recombinant RSV G proteins and affinity measurements

The extracellular domain of the G protein was generated by RT-PCR from RNA isolated from HEP2 cells infected with RSV-A2 or RSV-B1 strains.

For confirmatory ELISA assays, plates were coated with 0.2 μg/ml purified Ga protein in 0.1 M carbonate buffer (pH 9.6) overnight at 4°C and then blocked with PBS, 3% BSA. Culture supernatants were transferred and incubated at 25°C for 1 h; then the plates were washed and incubated with goat anti-human Fcγ fragment-specific Ab conjugated to HRP (Jackson ImmunoResearch) for 1 h. Chemiluminescent substrate (Pierce) was added, and luminescence was detected on a PE Victor plate reader.

Recovery of Gab-positive Abs

Total mRNA from positive limiting-dilution wells was extracted using a RNA purification kit (RNasy; Qiagen). RT-PCR was performed using total RNA preparations and oligonucleotides as primers. For amplification of a priori unknown V gene rearrangements, a collection of family-specific V gene primers was constructed, which recognizes all functional V gene segments in the human Igκ locus. A mixture of 5’ V gene primers were used together with primers specific for the Cκ, Cε, and Cα constant regions. The Qiagen OneStep RT-PCR kit was used for amplification. In the coupled RT-PCR reactions, cDNA was synthesized with a blend of RT enzymes (Omniscript and Senscript) using antisense sequence-specific primers corresponding to Cκ, Cε, or a consensus of the CH1 regions of Cγ genes. RT was performed at 50°C for 1 h followed by PCR amplification of the cDNA by HotStar TaqDNA polymerase for high specificity and sensitivity. PCR reactions were run at 95°C for 15 min, initial hot start followed by 20 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s (annealing), and 72°C for 1 min (elongation). For the second round of amplification, an aliquot of 5 μl of the first amplification reaction was used. These primers carried 5’-BglII and 3’-XbaI restriction sites. Thirty PCR cycles were performed per round with identical conditions for the first and second rounds of amplification. For each reaction, 5 μl were separated on a 1% agarose gel and stained with ethidium bromide. The V-C PCR product was predicted to amplify rearranged fragments of 500 and 450 bp. PCR bands with a molecular size of ~500 bp indicated a positive result. PCR products were purified (Qiagen gel purification kit), and extracted PCR products were sequenced using specific constant region primers. Sequences were analyzed by VBASE2 to identify germline V(D)J, Ig CDR3 length, and somatic hypermutations.

Each PCR fragment was digested and cloned into an Ig expression vector carrying the constant region of human V1, Cε or Cα, respectively, for in vitro Ab production in a mammalian expression system. Each Ab was transiently produced in HEK293 cells and purified by chromatography on immobilized protein A (Aragen Bioscience).
The recombinant proteins were cloned as 6× His tag fusion proteins in the mammalian expression vector pSecTag2 hygro A (Invitrogen), inserted directly downstream of the mouse IgG signal peptide. For codon optimization, viral codons were replaced by those most frequently used in human cells (36). Optimization of the sequence was performed by GenScript based on the amino acid sequence of GenBank database entry NC_001781. The CMV-IE intron A was added into both vectors by replacing the CMV promoter of pSecTag2 plasmid. All RSV recombinant plasmids were confirmed by DNA sequencing.

For protein production (Aragen Bioscience), HEK-293 cells adapted to serum-free growth medium were seeded at 1 × 10⁶ cells/ml in 1 liter and placed on a shaking platform 1 day before transfection. Transfection was accomplished using 293fectin (Invitrogen) mixed with 1 mg of plasmid DNA. Supernatant from transfected cells was harvested 5 days post-transfection and analyzed by SDSD-gel electrophoresis with Coomassie Blue staining (37). For immunoblot analysis, the protein was transferred from the gel to a nitrocellulose membrane. Bound Ab was detected with peroxidase-linked goat anti-human IgG, via ECL by standard methods (38).

Relative affinities were initially determined by ELISA using a fixed recombinant Ga protein concentration of 1 µg/ml on the plate and varying Ab concentrations. The affinity of several of the most promising Abs was measured by Biacore analysis (Biosensor Tools).

**Virus stocks and virus binding assay**

Viruses were obtained from ATCC (A Long, B 18537), Dr. Ralph Tripp (University of Georgia; strain A2), and Meridian Bioscience (strain Memphis 37) and propagated in HEP2 or HeLa cells (both from ATCC), as previously described (39). Plates were coated with viral preparations at 10⁶ PFU/well in 100 mM carbonate buffer at pH 9.6 overnight at 4°C and blocked in 5% milk in PBS-Tween for 1 h at 25°C. Serial dilutions of Abs were added to wells in blocking buffer for 1 h at 25°C. Binding was detected using goat anti-human Fcγ-HP (1/2000 dilution; Jackson ImmunoResearch), and tetramethylbenzidine substrate turnover was measured at 450 nm.

**Virus neutralization measurement**

Ab-induced neutralization of virus in vitro was determined by a standard plaque assay. HEP2 cells were plated in 12-well plates at 2 × 10⁴ cells/well. Approximately 200 PFU of RSV per well were added to dilutions of Abs in the presence of rabbit complement serum (Sigma-Aldrich) for 1 h at 25°C. The Ab-virus mixture was then added to HEP2 cells at 200 µl/well for 2 h at 37°C to allow for infection. After this infection period, supernatant was removed, and medium containing 1% methylocellulose was added to all wells. Plates were incubated at 37°C for 6 days; then cells were fixed and stained for plaque number determination as follows. Methylocellulose was aspirated, and cells were fixed in 100% methanol for 30 min at room temperature. The plates were washed three times with PBS, 5% milk. Goat anti-RSV Ab (Chemicon) was added (1/500 dilution) in PBS, 5% milk for 1 h. Plates were washed three times with PBS, 5% milk. ImmunoPure rabbit anti-goat IgG (H+L) peroxidase-conjugated secondary Ab (Thermo Scientific) was added at 1/500 dilution in PBS, 5% milk for 1 h. Plates were washed three times with PBS. Plaques were visualized by adding 1-Step chloronaphthol substrate (Pierce), 200 µl/well for 10 min. Plates were rinsed with water and allowed to air dry. Plaques were counted in each well by manual counting.

Activity was also measured using A549 cells, a polarized type II human lung epithelial cell line (ATCC). For this study, cells were plated at 1 × 10⁵ cells/well, and the dose of virus (strain A2) was adjusted to a 10-fold lower multiplicity of infection than that used for HEP2 cells.

**In vivo treatment and measurement**

Prophylaxis and treatment studies in mice were conducted at Aragen Bioscience in accordance with procedures approved by their Institutional Animal Care and Use Committee. Female BALB/c mice, 6–8 wk old, were infected intranasally at day 0 with 10⁶ PFU of RSV Long strain virus. Ab was injected i.p. at either day 1 (prophylactic) or day +1 or +3 or +5 after virus inoculation (treatment), using doses varying from 1.5 µg/kg to 15 mg/kg of Ab for the prophylactic study; for the treatment study, all Abs were used at 5 mg/kg. Viral load for the prophylactic study was quantified as PFU per gram of lung tissue at day 5. For the treatment study, viral load was determined at 2 or 4 days after treatment by and quantitative PCR after extracting RNA from lung tissue using a Trizol extraction kit (Invitrogen).

**Results**

**Single B cell phenotyping**

PBMC were isolated from 24 human adult subjects 1–4 mo after PCR confirmation of RSV infection. B cells were isolated and transfected using EBV to stimulate proliferation and secretion (33). From our screen of >20 million primary B cells, 12 clones were recovered that met stringent specificity criteria described below. CellSpot, a multiplexed single-cell phenotyping technology that enables high throughput and high content screening, has been recently described (35). The assay principle is illustrated in Fig. 1A with representative images shown at low and high magnification in Fig. 1, B and C. The high sensitivity and high throughput of the method enabled identification and recovery of rare favorable clones. RT-PCR was then used to recover the Ab sequences. Because the entire process from PBMC to cloned cDNA required only 14 days to perform, nearly the entire B cell repertoire could be screened before EBV-stimulated Ab secretion declined.

The stringent specificity criteria enabled by the multiplexed screen involved using three primary screen probes conjugated to three distinguishable bead types: G protein from two divergent RSV strains (Ga, Gb); and the more conserved F protein. The anti-RSV repertoire of 24 subjects is summarized in Fig. 2. The histogram shows the frequencies of B cells specific for F, Ga, Gb, and both Ga and Gb (Gab, in red) for each subject. Of the 24 donors, only 3 had the desired Gab phenotype at >20 B cells per million. Even among these exceptional donors, the frequency of the desired B cells was generally <50 per million, a frequency >10-fold lower than for B cells that recognized any RSV Ag but did not meet the stringent specificity criteria. For the entire cohort,
the frequency of Gab-positive B cells was <10 per million. Further, focusing only on subjects with the highest frequency of B cells meeting the selection criteria would have resulted in the loss of the highest affinity clone, 3D3, which came from study subject 5 for whom the frequency of positive B cells was 18 per million.

The frequency of Ag-specific B cells declines with time. We conducted a similar survey of 10 donors with a history of confirmed RSV infections 9–12 mo before B cell screening. Consistent with prior reports of poor immunological memory to RSV (40) as well as with the natural decline of Ag-specific memory B cells, the frequency of anti-RSV B cells in these samples was ~10-fold lower than for recently infected donors (data not shown).

Cloning and characterizing anti-Gab Abs

Following an initial multiplexed survey to define the frequency of B cells producing Abs with specificity for G protein from both RSV A and B strains, but no binding to F, we cloned the Gab-reactive Abs by transforming another aliquot of B cells from selected subjects, plating the cells at 300 B cells per well, and performing a single-cell phenotyping assay after 6 days. Twelve wells that contained B cells secreting anti-Gab Ab were selected for limiting-dilution cloning. From each positive limiting-dilution cell clone, Ig H chain and corresponding Ig L chain transcripts were amplified by reverse transcriptase nested PCR and cloned into eukaryotic expression vectors to produce human mAbs of the same specificity in vitro.

Analysis of the H chain repertoire within our suite of Abs showed that three VH families were used. The VH1, VH2, and VH3 family were used by 2, 4, and 6 Abs, respectively. Sequences in the variable regions of both heavy and light chains were mutated in-frame without stop codons, as expected for genotypically normal memory B cells. All sequences had unique VDJ joining regions (data not shown), with the number of mutations in the Ig genes ranging from 4 to 40 per sequence for an overall mutation frequency of 5%. This range of deviation from germ-line is consistent with an Ag-driven immune response (41). Although mutations were detected in the framework regions, most differences were within the CDRs, consistent with other studies of Ab-Ag binding (42). Similar restriction in gene usage has been reported for Abs against other viruses, including rotavirus (43) and hepatitis C (44).

Recombinant Abs were confirmed as Gab reactive by ELISA. Western blot analysis using a lysate of RSV strain A2 or purified Ga protein suggested that many of the Abs recognized linear epitopes. The antigenic determinants of these Abs were mapped using a set of overlapping 12-mer peptides derived from the RSV Ga protein sequence, each shifted by 1 residue. Of the 12 cloned Abs, 9 reacted with linear epitopes. As shown in Fig. 4A, Ab 3D3 reacted strongly with 4 consecutive peptides spanning residues 164–172, thereby mapping the 3D3-binding domain to residues 164–172 (HFEVFNFVP). The epitopes of representative Abs are shown in Table I. In addition, we found three mAbs with Gab specificity that likely recognize conformational epitopes given that they failed to recognize any linear peptide.

Although the discovery method was unbiased with regard to where on the G protein the Ab bound, as long as the Ab bound both Ga and Gb, all of the mapable Abs recognized epitopes within a few amino acids of each other. This region was previously known to be conserved across RSV strains (11). We have confirmed and extended this finding by collating data on 37 published RSV G protein sequences. The polymorphism frequency per residue, plotted in Fig. 3B, revealed absolute conservation at the site to which our Abs bind. The only other highly conserved regions were smaller segments at the transmembrane region and the alternative start site that results in a secreted form of the G protein.

Ab-mediated neutralization of secreted factors is generally more efficient with relatively high-affinity Abs (45). The affinity of several of the most promising Abs was measured by Biacore analysis using purified recombinant RSV G proteins. Binding and dissociation profiles for Abs 3G12 and 3D3 are shown in Fig. 4. The ratio of off- to on-rates for 3D3 yielded affinities of 1 pM for Ga and 3

FIGURE 2. Survey of memory B cells from 24 donors with PCR-confirmed RSV infection. The histogram shows frequency in parts per million of B cells specific for various Ags: Ga (pink); Gb (yellow); both Ga and Gb (Gab, red); F (blue). Desired cells were the Gab, strain-independent, Abs to G.

FIGURE 3. A, Epitope mapping of mAb 3D3 against peptides from RSV G protein. An array of peptides, 12 amino acids long in a staggered sequence, was probed with Ab. The common epitope for clone 3D3, highlighted in blue, is HFEVFNFVP. B, The set of peptides that the Gab cross-reactive human Abs bind is coincident with the most highly conserved region of the RSV G protein. Aas 160–186 are shown with the invariant region underlined and the conserved disulfide pairings in red. A survey of 37 published G protein sequences from diverse strains was collated to plot the number of variants at each residue (a value of 1 means that no other variants were found).
pM for Gb, and for 3G12 affinities of 579 pM for Ga and 173 pM for Gb. The affinity and epitope recognition characteristics of representative Abs from the suite, along with H and L chain subtypes, are summarized in Table I.

Biological activity of the novel Abs

All of our primary screening was performed with G protein purified from viral lysates. The resulting Abs also reacted with immobilized virus preparations, as shown in Fig. 5A, with good correlation between binding to the isolated protein and to immobilized virus, as shown in Fig. 5B.

Two of the novel Abs have been tested for biological activity in comparison with palivizumab. The new Ab variable domains were cloned into a human IgG1 Fc constant domain, the same as palivizumab. The new Ab variable domains were determined by Biacore analysis as illustrated in Fig. 4.

A potency advantage was also seen in a prophylactic mouse model, as shown in Fig. 7A. For this experiment, mice were treated with Ab by a single i.p. injection at day −1 and then infected intranasally at day 0. Viral load was determined by measuring PFUs at day +5. 3D3 was significantly (p < 0.05) more effective than palivizumab for the two lowest doses; however, 3G12 was not significantly different.

For prophylaxis, in vitro assays provide a reasonable mimic of the in vivo condition because the Ab is already present in the lungs before infection. For the treatment setting, however, there is a complex interaction between the virus and the host immune response (15), suggesting that properties other than affinity and in vitro potency may also be critical to the in vivo response. For example, the substantially higher affinity of motavizumab than of palivizumab has not provided a major improvement in efficacy (6, 7).

In preparation for the treatment study, we examined the time course of the response. As shown in Fig. 7B, viral load peaked at day +5. We also observed a high correlation between PFUs and quantitative PCR of viral transcript (r2 = 0.85); the latter provided a more sensitive readout as virus levels declined and was therefore used for the treatment study. Mice were infected intranasally at day 0 and treated with a single i.p. injection of Ab at day +3 at a dose that was fully protective in the prophylactic model (5 mg/kg). As shown in Fig. 7C, both anti-G Abs were markedly effective at reducing viral load at days +5 and +7 postinfection, whereas palivizumab was less effective. Specifically, 3G12 was significantly more active (p < 0.05) than palivizumab at both days +5 and +7, as was 3D3 compared with palivizumab (p = 0.076) at day +5 and at day +7 (p = 0.044). Similarly, treatment at day +5 and assay at day +7 showed significantly (p < 0.05) higher activity for both of the anti-G Abs than that of palivizumab.

Discussion

RSV is comparable with influenza in its medical impact. Unlike influenza, for which vaccination is quite effective, RSV has been refractive to vaccine approaches. For this reason, we chose to find a mAb potentially suitable for use as a therapeutic against RSV. Human-derived mAbs have been recognized as a promising source of therapeutics since the earliest days of hybridoma technology (16), with utility for treatment of several human diseases, including RSV, foreshadowed by experience with hyperimmune human globulins (47, 48).

Table I. Summary of key features of selected human Abs cross-reactive to the G protein of RSV from both A and B strains

<table>
<thead>
<tr>
<th>Clone</th>
<th>Epitope</th>
<th>Epitope Sequence</th>
<th>EC50a for Ga (pM)</th>
<th>Kd,b for Ga (pM)</th>
<th>Kd,b for Gb (pM)</th>
<th>Vh</th>
<th>Vk</th>
<th>Vλ</th>
</tr>
</thead>
<tbody>
<tr>
<td>3D3</td>
<td>164–172</td>
<td>HFEVFNVP</td>
<td>540</td>
<td>1.1</td>
<td>3</td>
<td>VH3–9</td>
<td>L6</td>
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</tr>
<tr>
<td>2B11</td>
<td>162–172</td>
<td>⏯HFEVFNVP</td>
<td>300</td>
<td>9</td>
<td>1.6</td>
<td>VHI–69</td>
<td>VL1–4</td>
<td></td>
</tr>
<tr>
<td>1G8</td>
<td>164–172</td>
<td>HFEVFNVP</td>
<td>730</td>
<td>24</td>
<td>141</td>
<td>VH4–39</td>
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<tr>
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<td>FEVFN</td>
<td>560</td>
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<td>378</td>
<td>VHI3–30.5</td>
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<tr>
<td>1D4</td>
<td>165–171</td>
<td>⏯ FEVFN</td>
<td>2570</td>
<td>230</td>
<td>52</td>
<td>VHI–46</td>
<td>B3</td>
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<tr>
<td>3G12</td>
<td>167–175</td>
<td>—VFNFVPCSIC</td>
<td>5710</td>
<td>579</td>
<td>173</td>
<td>VHI–39</td>
<td>L2</td>
<td></td>
</tr>
</tbody>
</table>

a Determined by ELISA as described in Materials and Methods.
b Determined by Biacore analysis as illustrated in Fig. 4.
The use of a high-throughput single-cell phenotyping technology, CellSpot, for discovery of candidate RSV-therapeutic mAbs from human blood cells has established a paradigm that should be broadly applicable. Overall, Abs produced by ~20 million individual human B cells were analyzed for three parameters, i.e., absence of binding to RSV F protein and strong binding to G protein from two dissimilar strains of RSV. Abs meeting these criteria recognized tightly clustered epitopes limited to ~20 residues in the conserved central cysteine-noose region. The frequency of such B cells overall was ~10 per million, ~10-fold lower than against other RSV epitopes. At this frequency, it would have been impractical to identify and recover the suite of clones by conventional methods. The affinity of the selected Abs was comparable or superior to the best murine hybridoma Abs of similar specificity (30, 49). At 1 pM, the Ab 3D3 has the highest affinity of all anti-G Abs described to date. It is likely that repeated exposure of adults to RSV over the course of decades may account for the presence of very-high-affinity mAbs in the human repertoire as compared with that of mice immunized for 6 to 8 weeks.

Modulation of the host immune system to virus infection is a common theme in virology. For example, the attachment factor of human metapneumovirus, another member of the Paramyxovirus family, is also poorly immunogenic, although it has no sequence homology to the RSV G protein (50). In the present instance, the conserved central region has been implicated in modulation of the host immune response (19–25). The low frequency of Abs against this functionally critical region has been reported to persist even after specific immunization with peptides from this region (51). Although the mechanism underlying the poor immunogenicity is unclear (17, 18), the fact remains that virus strains surviving in

FIGURE 5. A, Ab binding to immobilized live virus measured by ELISA. B, Rank order by EC_{50} values calculated from the data closely matched the ranking by biosensor-derived affinity to recombinant Ga protein (K_{d} values from Table I).

FIGURE 6. Antiviral activity of human anti-G mAbs 3D3 and 3G12 compared with the humanized murine anti-F Ab palivizumab. A, In vitro virus neutralization in the presence of complement for three laboratory strains (A2, A Long, B18537) along with a minimally passaged clinical isolate (Memphis (Mem) 37), all in HEp2 cells; the high-affinity anti-G Abs were more potent against all four representative virus strains than was palivizumab against Memphis 37. B, The same Abs were also more potent than palivizumab against RSV A2 in A549 cells, a polarized type II human lung epithelial cell line. Controls with no mAb were ~100 plaques/well in all cases.

FIGURE 7. Anti-viral activity in vivo. A, Murine prophylaxis. 3D3 and 3G12 were more potent than palivizumab at reducing viral load in the lungs. Mice were infected intranasally with RSV Long on day 0, following treatment at day −1 with the indicated dose by i.p. injection. Viral load was quantified as PFUs per gram of lung tissue at day 5. B, Time course of infection: high concordance between PFUs and quantitative PCR of viral transcript normalized against actin mRNA (quantitative (q) PCR). C, Murine postinfection treatment. 3D3 and 3G12 were more potent than palivizumab or a nonimmune isotype control at reducing viral load in the lungs. Mice were infected intranasally with RSV Long on day 0, followed by treatment at day +3 or day +5 by i.p. injection of a single 5-mg/kg dose. Viral load in lung tissue was quantified at the indicated times by quantitative PCR. Inset, Day +7 values on a magnified scale.
nature are those that avoid inducing an effective Ab response to this region.

Support for potential therapeutic utility of targeting the G protein central conserved cysteine-noose region has recently been provided by direct comparison of murine monoclonal anti-F and anti-G Abs with low nanomolar affinities in a mouse treatment model (31), with superior performance demonstrated by the anti-G Ab for reducing both viral load and markers of airway inflammation. The results presented here further support the utility of an anti-G Ab in reducing viral load in both prophylactic and postinfection treatment mouse models. Accordingly, we hypothesize potential clinical benefit from supplying affinity-matured human Ab from adults to infants, who are less efficient at generating such Abs themselves (52). The novel Abs described here may provide attractive candidates for testing of this hypothesis in clinical trials.

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Disclosures

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