Generation and Characterization of ALX-0171, a Potent Novel Therapeutic Nanobody for the Treatment of Respiratory Syncytial Virus Infection

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Respiratory syncytial virus (RSV) is an important causative agent of lower respiratory tract infections in infants and elderly individuals. Its fusion (F) protein is critical for virus infection. It is targeted by several investigational antivirals and by palivizumab, a humanized monoclonal antibody used prophylactically in infants considered at high risk of severe RSV disease. ALX-0171 is a trimeric Nanobody that binds the antigenic site II of RSV F protein with subnanomolar affinity. ALX-0171 demonstrated in vitro neutralization superior to that of palivizumab against prototypic RSV subtype A and B strains. Moreover, ALX-0171 completely blocked replication to below the limit of detection for 87% of the viruses tested, whereas palivizumab did so for 18% of the viruses tested at a fixed concentration. Importantly, ALX-0171 was highly effective in reducing both nasal and lung RSV titers when delivered prophylactically or therapeutically directly to the lungs of cotton rats. ALX-0171 represents a potent novel antiviral compound with significant potential to treat RSV-mediated disease.

Human respiratory syncytial virus (RSV) is the most important viral pathogen causing acute lower respiratory tract infections in infants worldwide and is estimated to result in ∼3.4 million yearly hospitalizations and ∼200,000 deaths globally (1). RSV typically causes its primary infection at the point of entry: apical ciliated epithelial cells that line the nasal cavity and airways (2, 3). Primary infections are usually asymptomatic, with clinical signs ranging from mild upper respiratory tract illness to severe lower respiratory tract infections, including pneumonia and bronchiolitis (4). In addition to the acute consequences of infection, the development of long-term recurrent wheezing and asthma has been associated with severe RSV infections in infancy (5, 6). Despite the major clinical importance of RSV, no vaccines or widely accepted antiviral therapies are currently available. The only available drug specific for human RSV is palivizumab (Synagis), a marketed monoclonal antibody that is administered prophylactically before and during the RSV season to infants at high risk of having severe human RSV disease (7–9). Its use is restricted to premature infants (gestational age, <29 weeks), if they have no other underlying morbidities, and infants with chronic lung disease, congenital heart disease, or a compromised immune system during the first year of life (10).

RSV is a member of the Pneumovirus genus of the Paramyxoviridae family and has a linear single-stranded, nonsegmented RNA molecule of negative polarity as its genome. This genome contains 10 genes which encode 11 proteins. The transmembrane glycoproteins F and G are the primary surface antigens of RSV. The attachment (G) protein mediates binding to cell receptors, while the F protein promotes fusion of the viral and cell membranes, allowing virus entry into the host cell cytoplasm (11). The F protein also promotes the fusion of infected cells with adjacent uninfected cells, facilitating the formation of multinucleated cell formations (syncytia), which allow cell-to-cell transmission of the replicated viral RNA and confer additional protection for the virus against host immune responses (12).

On the basis of the antigenic and genetic variability of the G protein, two subgroups of RSV (subgroups A [RSV-A] and B [RSV-B]) have been identified, and these are composed of evolving genotypes (13–17). In contrast to the G protein, the F protein is mostly conserved between RSV subgroups A and B (89% amino acid identity) and is therefore considered the most promising target for the development of viral entry inhibitors.

Nanobodies are therapeutic proteins derived from the heavy-chain variable domains (VHH) that occur naturally in heavy chain-only immunoglobulins of the Camelidae (18, 19). The formatting flexibility of Nanobodies into multivalent constructs, their small size, their stability (which allows delivery through nebulization), and their ease of production make their use against viral targets appealing (20–22).
Here we describe the in vitro and in vivo characterization of ALX-0171, a trivalent Nanobody composed of three monovalent Nb017 moieties linked together with glycine-serine (GS) linkers which is designed to target the RSV F protein for delivery via inhalation. ALX-0171 is currently in clinical development for the treatment of RSV infections in infants (23).

**MATERIALS AND METHODS**

**Generation of RSV-specific Nanobodies.** Monovalent RSV F protein-specific Nanobodies were identified from immune libraries of llamas that received repetitive injections with soluble recombinant F protein (Fprep, NIH protein), inactivated RSV-A (catalog number RSV79; HyTest), or a combination of both antigens. The soluble recombinant form of the F protein (derived from the Long strain) was produced with recombinant Sendai virus in embryonated eggs (24). RSV-neutralizing Nanobodies were identified by screening 162 distinct clones in an in vitro microneutralization assay with RSV subtype A Long on HEp-2 cells. RSV-neutralizing Nanobodies were formatted into multivalent constructs by genetic fusion with flexible Gly-Ser (GS) linkers of different lengths. Monovalent RSV Nanobody was routinely generated as C-terminal c-myc- or anti-His-tagged proteins in *Escherichia coli* (22). For the production of untagged monovalent and trivalent Nanobody constructs, a *Pichia pastoris* X-33 expression system was used (see Fig. S1 in the supplemental material). ALX-0171 is a trimeric Nanobody consisting of three RSV-targeting Nb017 subunits linked by two GS linkers.

**Competition enzyme-linked immunosorbent assay (ELISA).** Ninety-six-well microtiter plates (MaxiSorp immunoplates; Nunc) were coated overnight with inactivated RSV-A (HyTest) at 3.5 μg/mL in bicarbonate buffer. The plates were blocked for 1 h with SuperBlock T20 (phosphate-buffered saline [PBS]; blocking buffer) (Pierce) before being washed 3 times with PBS–0.05% Tween 20. Dilutions of ALX-0171 or palivizumab were mixed with 10 μM biotinylated ALX-0171 (1:1 ratio) and immediately transferred to the plates. After overnight incubation at room temperature (RT) and appropriate washing, a 1/3,000 dilution of peroxidase-labeled streptavidin (Thermo Scientific) was added to the plates for 1 h at RT. Bound streptavidin-horseradish peroxidase was revealed by adding 100 μL/well of enhanced soluble tetramethylbenzidine (esTMB; SDT Reagents) for 10 min, followed by 1 N HCl. The absorbance was read at 450 nm, and the reference wavelength was 620 nm.

**SPR.** Affinity constants (the equilibrium dissociation constants [K
\text{D}]) of Nb017, ALX-0171, and palivizumab were determined by surface plasmon resonance (SPR) analysis on a Biacore T200 instrument (GE Healthcare) was amine coupled to a CM5 sensor chip at a density of 12,000 to 13,000 resonance units (RU) in 10 mM acetate buffer, pH 4.5. Approximately 70 RU of His-tagged prefusion F protein was then coated overnight with inactivated RSV-A (HyTest) at 3.5 μg/mL in bicarbonate buffer. The plates were blocked for 1 h with SuperBlock T20 (phosphate-buffered saline [PBS]; blocking buffer) (Pierce) before being washed 3 times with PBS–0.05% Tween 20. Dilutions of ALX-0171 or palivizumab were mixed with 10 μM biotinylated ALX-0171 (1:1 ratio) and immediately transferred to the plates. After overnight incubation at room temperature (RT) and appropriate washing, a 1/3,000 dilution of peroxidase-labeled streptavidin (Thermo Scientific) was added to the plates for 1 h at RT. Bound streptavidin-horseradish peroxidase was revealed by adding 100 μL/well of enhanced soluble tetramethylbenzidine (esTMB; SDT Reagents) for 10 min, followed by 1 N HCl. The absorbance was read at 450 nm, and the reference wavelength was 620 nm.

**Statistical analysis.** A one-way analysis of variance (ANOVA) followed by a post hoc Dunnett’s test for pairwise comparison was performed to compare the treated groups and the buffer-treated groups. A P value of <0.05 was considered statistically significant. Statistical analysis was performed with GraphPad Prism (version 5) software.

**RESULTS**

**Generation and production of ALX-0171.** The development of a *Pichia pastoris* strain for the production of ALX-0171 delivered a stable recombinant strain suitable for the manufacture of the multivalent trimeric ALX-0171 Nanobody. Further streamlining of the typical *Pichia pastoris* fermentation process resulted in an
ALX-0171 upstream process in which more than 7.5 g/liter ALX-0171 was secreted in the fermentation broth. After clarification of the broth, the downstream process consisted of capture, intermediate purification, and polish chromatography steps and was followed by a final formulation step. A range of ALX-0171 concentrations was tested in combination with a series of buffers and excipients to provide the highest stability after storage, nebulization, freeze-thaw, etc. These formulation and (stressed) stability studies resulted in the formulation of ALX-0171 as a stable nebulizer solution.

**Characteristics of ALX-0171 binding to RSV F protein.** The kinetics of Nb017 and ALX-0171 binding to the RSV F protein in its prefusion conformation was assessed by SPR analysis. The conformational integrity of the F protein was confirmed with the antigenic site Ø-specific Fab D25, which bound only to the prefusion conformation, whereas the antigenic site I-specific Fab 2F did not bind to the prefusion conformation, as expected. The binding affinity of palivizumab was 0.88 nM on the prefusion conformation, whereas the antigenic site Ø-specific Fab D25, which bound only to the prefusion conformation titers (as determined by endpoint dilution assays) are shown in Table 2. ALX-0171 was \( \approx 180 \)-fold more potent than palivizumab against the RSV-A clinical isolates tested (range, 180- to 409-fold) and \( \approx 12 \)-fold more potent than palivizumab against the RSV-B clinical isolates tested (range, 11.5- to 647.5-fold), and this potency difference was in the same range as that determined for the prototypic RSV-A Long and RSV-B 18537 strains (177- and 17-fold, respectively) using the same assay.

To provide a more comprehensive understanding of the relative neutralization capacity of ALX-0171 and palivizumab against a large panel of RSV clinical isolates, 61 RSV isolates, including the RSV Tracy and RSV-B 18537 strains (see Table S1 in the supplemental material), were tested for inhibition by ALX-0171 and palivizumab at a single concentration of 40 \( \mu \)g/ml in a semiquantitative plaque reduction assay. This concentration represents the mean 30-day trough serum concentration of palivizumab after the first intramuscular injection (32). ALX-0171 and palivizumab reduced the viral titers by 2 \( \log_{10} \), for 97% and 85% of viruses tested, respectively (Table 3). For RSV-A isolates, this proportion was increased by 2 log units and by 0.6 log units for RSV-B 18537.

**FIG 1.** Microneutralization assay with ALX-0171, Nb017, and palivizumab. The capacities of ALX-0171, Nb017, and palivizumab to neutralize RSV-A Long (A) and RSV-B 18537 (B) were tested. The results shown depict the means of triplicate values \( \pm \) SEs. OD, optical density.

**Table 1.** Affinities of ALX-0171, Nb017, and palivizumab toward RSV prefusion F protein measured by SPR

| Compound          | \( k_{\text{on}} \) (M\(^{-1}\) s\(^{-1}\) [10\(^8\)]) | \( k_{\text{off}} \) (s\(^{-1}\) [10\(^{-4}\)]) | \( K_D \) (nM) | \( R_{\text{max}} \) (RU) | \( \chi^2 \) (RU\(^2\)) |
|-------------------|-----------------|-----------------|-------------|----------------|-----------------|
| Nb017             | 1.305           | 233.5           | 17,880      | 14.73          | 0.713           |
| ALX-0171          | 4.091           | 4.604           | 0.113       | 24.30          | 0.46            |
| Palivizumab       | 0.084           | 0.739           | 0.879       | 56.2           | 0.0947          |
| Fab D25           | 0.488           | 0.331           | 0.068       | 51.78          | 0.158           |
| Fab 2F            | >20             |                 |             |                |                 |
100% with ALX-0171 and 84.4% with palivizumab ($P = 0.053$), and for RSV-B isolates, this proportion was 93.1% with ALX-0171 and 86.2% with palivizumab ($P = 0.67$). These results are consistent with published data demonstrating that palivizumab neutralized 75/77 (97%) clinical isolates tested (32). Importantly, ALX-0171 demonstrated a broad RSV strain neutralization capacity that was at least as extensive as that of palivizumab when looking at 2 log10 reductions.

Our data were the most striking when the complete suppression of RSV replication was compared between ALX-0171 and palivizumab. When they were tested at equivalent concentrations, complete virus inhibition was defined as no detectable virus plaques. The limit of virus detection in the plaque reduction neutralization assay was 5 PFU/ml.

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Furthermore, no effect of nebulization on ALX-0171 potency was observed by ELISA analysis (see Table S2 in the supplemental material).

Local delivery of ALX-0171 to cotton rats reduces the RSV load in the nose and lung. Cotton rats were used to evaluate the in vivo efficacy of ALX-0171 against RSV. Different doses of ALX-0171 were administered via the intranasal route on different days after RSV Tracy challenge either once (day 2 or day 3) or twice (day 2 and day 3) or by nebulization 1 h before RSV Tracy challenge (Table S1). A dose-dependent reduction in viral titers was seen in the lungs, which was significant for the two higher doses (P = 0.01 and 0.006 for doses of 0.8 mg/kg and 2 mg/kg, respectively). Furthermore, RSV replication was almost completely blocked in the lungs, even at the lowest dose tested (>3.67 log10 reduction, P < 0.0001).

**DISCUSSION**

ALX-0171 is a novel therapeutic biologic in development for the treatment of RSV infections in infants. ALX-0171 is a trimeric Nanobody that binds an epitope in antigenic site II of RSV F protein with subnanomolar affinity. This epitope partially overlaps the palivizumab epitope.

The formatting of the monovalent Nb017 into the trivalent Nanobody ALX-0171 increased the potency of neutralization (>6,000-fold) of both RSV-A and RSV-B strains. Although formatting clearly increased the binding affinity for the biologically relevant prefusion conformation of the F protein (~160-fold), it does not fully explain the observed large increase in the virus neutralization capacity of ALX-0171. Our results are consistent with those of a previous report demonstrating that a bivalent Nanobody construct specific for F-protein antigenic site II had a 4,000-fold improved neutralization capacity compared to that of the monovalent construct against RSV-A Long (22). Likewise, the difference in potency between palivizumab Fab and the full-length antibody is 100-fold, while there is only a minimal improvement

**TABLE 4** Binding reactivity of Nb017 to membrane extracts of cells infected with distinct RSV-A Long escape mutants

| RSV mutant | Amino acid substitution(s) | Antigenic site | % Nb017 binding | % Nb017 binding |
|------------|-----------------------------|----------------|-----------------|-----------------|
| R47F/4     | N262Y                        | II             | 1.4 ± 1.6       | 100 ± 20        |
| R47F/7     | N268I                        | II             | 61.1 ± 17.2     | 100 ± 20        |
| RAK13/4    | N216D/N262Y                  | II             | 0.6 ± 0.8       | 100 ± 20        |
| R7C2/11    | K272T                        | II             | 19.5 ± 16.8     | 100 ± 20        |
| R7C2/1     | K272E                        | II             | 0.9 ± 1.1       | 100 ± 20        |
| R7.936/1   | V447A                        | IV             | 119.9 ± 37.3    | 100 ± 20        |
| R7.936/4   | K433T                        | IV             | 122.3 ± 40.5    | 100 ± 20        |
| R7.936/6   | I432T                        | IV             | 106.5 ± 15.9    | 100 ± 20        |
| R9.432/1   | S436F                        | IV             | 121.4 ± 20.0    | 100 ± 20        |
| RRA3       | N262Y/R429S                  | II and IV      | 1.6 ± 3.2       | 100 ± 20        |

a Absorbance results were normalized to those for a reference Nanobody recognizing antigenic site I (191C7) with preserved binding to all depicted mutants to account for the difference in F-protein expression. Nb017 was used at 0.2 μg/ml.

b Data represent the percent binding to that of the reference RSV-A Long wild-type strain ± standard deviation. Shading and boldface indicate where the binding of Nb017 was >75%, shading indicates where the binding of Nb017 was 25 to 75%, and no shading indicates where the binding of Nb017 was < 25%.

**FIG 3** Results of competitive-binding ELISAs. The inhibition concentration-response curves obtained when biotinylated ALX-0171 was incubated with increasing concentrations of either unlabeled ALX-0171 or palivizumab are shown. The results shown depict the means of triplicate values ± SEs.
in the $K_T$ (33). However, the basis of this observation is not yet understood, and the precise mechanisms involved remain under investigation.

Like palivizumab, Nanobodies Nb017 and ALX-0171 bind to both the postfusion and prefusion conformation of the F protein and, as a result, likely inhibit the conformational changes related to F-protein activation, as suggested for other Nanobodies and monoclonal antibodies specific for antigenic site II (21, 34–36). This hypothesis is supported by a model of the RSV F-protein structure, in which the residues of antigenic site II are proximal to sequences that form the central prefusion structure, in which the residues of antigenic site II are extended complementarity-determining region loops may enhance the accessibility to F-protein antigenic site II (37, 38), (ii) the GS linker is likely more flexible than the hinge of a full-length human immunoglobulin, screened for high RSV neutralization capacity: (i) the smaller size of ALX-0171 and its extended complementarity-determining region loops may enhance the accessibility to F-protein antigenic site II (37, 38), (ii) the GS linker is likely more flexible than the hinge of a full-length antibody, and thus, ALX-0171 would be able to access its binding site more readily, and (iii) the trivalency of ALX-0171 may allow additional binding modes, such as simultaneous binding of three independent F-protein trimers or simultaneous binding of the three subunits within a single F-protein trimer (20, 21), as the length of the GS linkers was specifically designed to allow such interactions. Further crystallography studies are needed to elucidate the mechanisms involved in this increased neutralization capacity of ALX-0171.

**In vivo**, ALX-0171 was shown to be highly efficient at reducing and/or blocking RSV replication in the lung and nose, similar to what was reported for motavizumab, an affinity-matured version of palivizumab (39, 40), whereas no effect on viral load in the nose was demonstrated for palivizumab at doses of $\leq 15$ mg/kg (40, 41). There remains the possibility that the residual ALX-0171 present in the wash fluid interfered in the plaque assay. Nonetheless, despite this caveat, we believe that it remains the most valid assessment, as it reflects the fact either that less virus is present or that any virus still present is effectively neutralized. Importantly, as ALX-0171 is devoid of an Fc, its therapeutic effect of topical administration of purified human immunoglobulins, screened for high RSV neutralization

### TABLE 5 Reduction in RSV replication in cotton rats

| Expt no., route of delivery | ALX-0171 dose delivered (mg/kg) | Treatment regimen | Nasal titer Mean log$_{10}$ no. PFU ($\pm$ SD) vs buffer reduction vs buffer | Lung titer Mean log$_{10}$ no. PFU/g lung ($\pm$ SD) vs buffer reduction vs buffer |
|---------------------------|---------------------------------|------------------|-----------------------------------------------|-----------------------------------------------|
| Expt 1, intranasal        | Buffer                          | Days +2 and +3   | 4.78 (0.40) 1.32* 4.94 (0.13)                | 2.48 (0.56) 2.88* 2.68 (0.89) 2.00*          |
| 1                        |                                 | Days +2 and +3   | 3.46 (0.56) 1.29* 3.28 (0.40) 1.66*          | 3.06 (0.75) 2.37* 3.14 (0.53) 1.81*          |
| 2                        |                                 | Days +2 and +3   | 3.06 (0.75) 2.17* 3.14 (0.53) 1.81*          | 2.53 (0.88) 2.26* 3.18 (0.43) 1.76*          |
| 4                        |                                 | Days +2 and +3   | 1.68 (0.28) 3.10* 2.05 (0.66) 2.89*          | 2.00 (0.72) 2.84* 2.04 (0.47) 3.15*          |
| 21                       |                                 | Days +2 and +3   | 4.84 (0.15) 5.19 (0.24)                      | 4.88 (0.34) 1.41 (0.07) 2.64 (0.34)          |
| Expt 2, intranasal        | Buffer                          | Days +2 and +3   | 2.00 (0.72) 2.84* 2.04 (0.47) 3.15*          | 1.73 (0.21) 3.31* 2.55 (1.13) 2.64*          |
| 4                        |                                 | Days +2 and +3   | 1.54 (0.59) 3.30* 1.88 (0.50) 3.30*          | 3.05 (0.59) 2.31 (0.49) 1.30 (0.43) 1.30*    |
| 19                       |                                 | Days +2 and +3   | 2.35 (0.88) 3.30* 2.04 (0.47) 3.15*          | 1.73 (0.30) 3.07* 2.10 (0.32) 2.78*          |
| 68                       |                                 | Days +2 and +3   | 4.81 (0.36) 4.88 (0.34)                      | 1.84 (0.38) 2.97* 2.10 (0.22) 2.78*          |
| Expt 3, intranasal        | Buffer                          | Days +2 and +3   | 4.53 (0.87) 2.28 (0.60) 2.60*                | 4.35 (0.87) 2.28 (0.60) 2.60*                |
| 4                        |                                 | Day +2           | 2.88 (0.89) 2.00*                             | 2.88 (0.89) 2.00*                             |
| 5                        |                                 | Day +3           | 2.10 (0.22) 2.78*                             | 2.10 (0.22) 2.78*                             |
| Expt 4, nebulization      | Buffer$^{a}$                    | Hour -1          | 5.04 (0.25) 5.05 (0.22)                       | 5.04 (0.25) 5.05 (0.22)                       |
| 0.3$^{b}$                |                                 | Hour -1          | 3.97 (0.62) 1.07 1.26 (0.34) 3.79*           | 3.97 (0.62) 1.07 1.26 (0.34) 3.79*           |
| 0.8$^{b}$                |                                 | Hour -1          | 3.02 (1.46) 2.02* 1.38 (0.43) 3.67*          | 3.02 (1.46) 2.02* 1.38 (0.43) 3.67*          |
| 2$^{b}$                  |                                 | Hour -1          | 2.87 (1.42) 2.18* 1.09 (0.05) 3.96*          | 2.87 (1.42) 2.18* 1.09 (0.05) 3.96*          |

$^{a}$ Cotton rats (6 to 8 rats per group) were challenged with $1.4 \times 10^5$ to $3.4 \times 10^5$ PFU of RSV Tracy (intranasally, 100 $\mu$L) on day 0. The animals received ALX-0171 at the indicated times either by intranasal administration or by nebulization. Viral titters and viral RNA loads in both the lung and nose were assessed on day 4 postinfection. $^*, P < 0.05$ versus buffer control. P values were determined by ANOVA with a follow-up Dunnett’s pairwise comparison test.

The delivered dose was estimated. The estimated delivered dose was defined as the estimated dose that is inhaleable by the cotton rats and was calculated using the following formula: delivered dose (in micrograms per kilogram of body weight) $= \frac{(aerosol concentration \times \text{minute volume} \times \text{treatment time} \times \text{percentage of particles of } \leq 5 \mu m \text{ (respirable fraction)})}{\text{minute volume}}$ 

Where the minute volume was estimated to be $0.7 \text{ liter minute/kg}$ (47) and the respirable fraction was estimated to be 80% on the basis of the characteristics of the nebulizer.
activity, resulting in a decrease in viral loads in cotton rats was shown to be 160 times greater than that by administration by the parenteral route (44). In addition to the greater effectiveness of the ALX-0171 administration route, the neutralization threshold of ALX-0171 (i.e., the expected lung concentration needed to exert the full antiviral effect) is also anticipated to be lower than that of palivizumab. This is particularly important in a clinical setting where the administered dose is a limiting factor.

In summary, ALX-0171 represents a novel, highly potent antiviral with broad specificity toward a large panel of RSV clinical isolates and may have significant potential for therapeutic use. Furthermore, direct delivery of ALX-0171 to the Airways/lungs by nebulization proved an effective mode of drug delivery, as even the lowest dose of only 1 mg/kg delivered intranasally still showed antiviral efficacy. As nebulization has been shown to result in fast and efficient drug delivery to the principal site of RSV infection (45), i.e., the upper and lower respiratory tract, this mode of drug delivery may provide major therapeutic advantages for ALX-0171 in treating RSV-infected patients.

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