Trivalency of a Nanobody Specific for the Human Respiratory Syncytial Virus Fusion Glycoprotein Drastically Enhances Virus Neutralization and Impacts Escape Mutant Selection

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ALX-0171 is a trivalent Nanobody derived from monovalent Nb017 that binds to antigenic site II of the human respiratory syncytial virus (hRSV) fusion (F) glycoprotein. ALX-0171 is about 6,000 to 10,000 times more potent than Nb017 in neutralization tests with strains of hRSV antigenic groups A and B. To explore the effect of this enhanced neutralization on escape mutant selection, viruses resistant to either ALX-0171 or Nb017 were isolated after serial passage of the hRSV Long strain in the presence of suboptimal concentrations of the respective Nanobodies. Resistant viruses emerged notably faster with Nb017 than with ALX-0171 and in both cases contained amino acid changes in antigenic site II of hRSV F. Detailed binding and neutralization analyses of these escape mutants as well as previously described mutants resistant to certain monoclonal antibodies (MAbs) offered a comprehensive description of site II mutations which are relevant for neutralization by MAbs and Nanobodies. Notably, ALX-0171 showed a sizeable neutralization potency with most escape mutants, even with some of those selected with the Nanobody, and these findings make ALX-0171 an attractive antiviral for treatment of hRSV infections.

Human respiratory syncytial virus (hRSV) is the most important viral pathogen causing acute lower respiratory tract infections (ALRI) in infants and young children worldwide (1, 2). hRSV is also an important cause of morbidity and mortality in the elderly and in adults with cardiopulmonary disease or with an impaired immune system (3–5). Despite its clinical relevance, neither vaccines nor widely used antiviral therapies are available against hRSV. In most cases, management of hospitalized patients is purely supportive.

hRSV is an enveloped nonsegmented negative-sense RNA virus, classified in the genus *Orthopneumovirus* of the newly created *Pneumoviridae* family within the order *Mononegavirales*, and thus standing apart from the original *Paramyxoviridae* family (6). The hRSV genome encodes 11 proteins, 2 of them being the major surface glycoproteins anchored into the virus envelope. These are (i) the attachment (G) protein, which mediates binding of the virus to the cell surface (7) and (ii) the fusion (F) protein, which promotes fusion of the virus and cell membrane to allow entry of the viral genome into the target cell as well as fusion of the membranes of infected cells with those of adjacent cells to form syncytia (8). While the G protein has no homology with the receptor-binding protein (HN or H) of paramyxoviruses, the F protein, despite limited sequence identity, shares structural features with the paramyxovirus F protein, suggesting that they all function through similar mechanisms (9).

The *Pneumoviridae* and *Paramyxoviridae* F proteins are homotrimeric type I fusion proteins that are incorporated into virus particles in a metastable conformation, called prefusion. Once these viruses are bound to the target cell, F is activated to initiate fusion of the viral and cell membranes (10). During this process, the F protein experiences a series of conformational changes which produce a highly stable structure, called the postfusion F protein (11). The hRSV F and G glycoproteins are the only viral gene products capable of inducing long-lasting protection in animal models against virus challenge (12–14).

There is compelling evidence that protection against hRSV infection is mediated mainly by neutralizing antibodies. For instance, passive transfer of serum neutralizing antibodies protects mice (15) and cotton rats (16) against hRSV challenge. Furthermore, a positive correlation has been found between high titers of serum neutralizing antibodies and protection of human volunteers against hRSV challenge (17), as well as protection of children (18) and the elderly (19) to natural hRSV infection. This evidence led to licensing in mid-1990s of an immunoglobulin preparation (RespiGam) made from donors screened for high titers of neutralizing antibodies against hRSV. Monthly prophylactic administration of RespiGam to children at high risk of having a severe hRSV infection reduced the frequency of hospitalization by about 55% and intensive care days by 97% (20, 21). However, since RespiGam had the disadvantage of requiring intravenous infusion, besides the theoretical risk of adventitious agents and possible interference with pediatric vaccines, it was soon superseded by palivizumab (Pz; Synagis), a humanized monoclonal antibody (MAb) that binds to the hRSV F protein (22–24). Pz is the only existing medicinal product specific for hRSV which, as done before with RespiGam, is used prophylactically in children at high risk of having severe hRSV disease (21, 25, 26).

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F and G glycoproteins are the targets of hRSV-neutralizing antibodies, but only MABs specific for hRSV F have been shown to be potent neutralizers *in vitro* of virus infectivity in the absence of complement (27). Several antigenic sites (I, II, and IV) were identified in the F molecule with murine MABs (28). Detailed structural features of site II and IV epitopes recognized by neutralizing MABs, and that are preserved in the pre- and postfusion conformations of hRSV F, were recently described (29–31). However, the majority of the neutralizing activity present in human Ig preparations could not be depleted with an immobilized soluble F protein folded in the postfusion conformation, suggesting that most human neutralizing antibodies recognize epitopes specific for the prefusion hRSV F protein (32). Indeed, new antigenic sites were found in soluble forms of hRSV F stabilized in the prefusion conformation which were recognized by highly neutralizing and prefusion specific MABs (33–35).

Camelids express not only conventional antibodies but also functional antibodies composed of only heavy chains (36). Nanobodies are recombinant therapeutic proteins based on the variable antigen-binding domains (designated VHH) of the *Camelid* heavy chain-only antibodies produced in either yeast or bacteria (the terms “Nanobody” and “Nanobodies” are registered trademarks of Ablynx NV). Nanobodies have been shown to be potent neutralizers of different viruses, including hRSV (37–39). Compared to conventional antibodies, Nanobodies are intrinsically stable molecules with improved solubility (40) and have the capacity to be nebulized without loss of physico-chemical properties or of potency (41). Due to their small size and extended CDR3 loops, Nanobodies can bind conformational epitopes that may be otherwise inaccessible to conventional antibodies. Their single-domain format greatly facilitates the construction of multivalent molecules by genetic linking using the same or different Nanobody building blocks. Thus, Nanobodies are attractive molecules for treatment of viral infections, such as hRSV.

ALX-0171 is a homotrivalent Nanobody comprising monovalent Nb017 directed to the hRSV F antigenic site II (41). ALX-0171 is currently being tested in hospitalized infants diagnosed with RSV lower respiratory tract infection (<https://www.clinicaltrials.gov/ct2/show/NCT02309320?term=ALX-0171&rank=4>). While the trivalent format highly increased the neutralization potency, compared to its monovalent counterpart, there is no information yet available about the effect of the multivalency on the frequency of *in vitro*-generated escape mutants or the type of mutants being selected. Since this information is important when assessing the clinical performance of these types of molecules, the following points were addressed here: (i) number of passages required for the emergence of escape mutants when hRSV is grown in the presence of suboptimal concentrations of either the monovalent (Nb017) or the trivalent (ALX-0171) Nanobodies, (ii) sensitivity of the respective RSV escape mutants to binding and neutralization by Nanobodies and previously described MABs that recognize epitopes of antigenic site II, and (iii) comparison of the selected mutations with those present in previously described escape mutants selected with MABs.

MATERIALS AND METHODS

Cells, viruses, and MABs. The Long strain (ATCC VR-26TM) of hRSV was used throughout this study. It was propagated in HEP-2 cells (ATCC CCL-23) grown in Dulbecco's modified Eagles's medium (DMEM) supplemented with 2.5% fetal calf serum and antibiotics (DMEM 2.5). The

virus was plaque purified by infecting HEP-2 cell monolayers with serial virus dilutions, followed by 5 days of incubation at 37°C in 0.7% Noble agar in DMEM 2.5. MABs used in this study have been described elsewhere (42, 43), as have the escape mutants of the Long virus selected with MABs (28, 44, 45). MABs were purified from ascites fluid by protein A-Sepharose chromatography, as described previously (42). Those used in enzyme-linked immunosorbent assays (ELISAs) were biotinylated by incubating 9/10 volume of MAB at 1.0 mg/ml in buffer containing 0.1 M Na₂CO₃, 0.15 M NaCl, pH 9.0 with 1/10 volume of *N*-hydroxysuccinimide biotin (catalog number H1759; Sigma) at 1 mg/ml in dimethyl sulfoxide for 2 h at room temperature, followed by dialysis against the same buffer.

Nanobodies. The RSV-neutralizing Nanobody Nb017 was identified from immune libraries of llamas, as previously described (37). The trivalent ALX-0171 Nanobody was constructed by PCR using genetic linking with two flexible Gly-Ser linkers to fuse the Nb017 subunits (Nb017-Nb017-Nb017) (41). Irrelevant Nanobodies against chicken lysozyme in monovalent and trivalent formats (IRR26 and IRR33) were included in the study as negative controls. Nanobodies were produced either in *Escherichia coli* with C-terminal c-myc-6×His tags (37) or in *Pichia pastoris* X-33 and purified as C-terminal c-myc-6×His-tagged or as tagless Nanobodies, as previously described (41).

Purified endotoxin-free Nanobodies were biotinylated with the EZ-Link Sulfo-NHS-LC-biotin labeling reagent (catalog number 21335; Thermo Scientific). Briefly, a 10-fold molar excess of EZ-Link Sulfo-NHS-LC-biotin was added to the Nanobodies and the mixture was incubated at 4°C for 2.5 h with gentle shaking. After labeling, Nanobodies were dialyzed with Slide-A-Lyzer G2 dialysis cassettes, 3,500 molecular weight cutoff (MWCO) membrane, 3-ml volume (catalog number 87723; Thermo Scientific) according to the manufacturer's instructions. Then, they were concentrated using a Vivaspin 6, 3,000 MWCO membrane (catalog number VS0691; Sartorius Stedim) for the monovalent Nanobodies and a Vivaspin 20, 5,000 MWCO membrane (catalog number VS2011; Sartorius Stedim) for the trivalent Nanobodies.

ELISAs for binding to extracts of hRSV-infected HEP-2 cells. HEP-2 cells growing in 100-mm petri dishes were infected with either wild-type (Long) or mutant hRSV viruses (1 to 3 PFU/cell). After 48 h, cells were harvested and extracts were made at 500 µl/plate of buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, and 1% octyl-glucoside).

Normalization of the amount of hRSV F protein in each cell extract was done in a sandwich ELISA as follows: MAB 2F was used to coat 96-well microtiter plates (400 ng/well in 50 µl phosphate-buffered saline [PBS] overnight at 4°C). The wells were blocked with 100 µl of 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature. After washing, 50-µl aliquots of duplicate serial dilutions of each cell extract in the same blocking solution were added to the plates and incubation was continued for 1 h. After washing, an excess of biotinylated 101F MAB (100 ng/well) was added and incubation continued for another hour. Finally, the bound antibody was visualized with 50 µl of streptavidin-peroxidase (catalog number RPN1231V; GE Healthcare) diluted 1:1,000 in blocking solution followed by 2% OPD (*o*-phenylenediamine dihydrochloride; catalog number P2903; Sigma). Reactions were stopped with 3 N H₂SO₄ before reading the optical density at 490 nm (OD₄₉₀). The dilution of each cell extract yielding the same OD value with MAB 101F (set at 90% of maximal signal) was chosen for subsequent binding assays.

The binding of different MABs or Nanobodies to infected cell extracts was assessed in a similar sandwich ELISA using MAB 2F to capture a set amount of each extract, determined as described in the previous paragraph. Binding of serial dilutions of biotinylated Nanobodies and MABs was allowed for 1 h, after which detection was done as described in the previous paragraph. The exception was binding of the commercially available humanized MAB palivizumab (Synagis; Medimmune) which, not being biotinylated, was detected with biotinylated sheep anti-human IgG (catalog number RPN1003V; GE Healthcare), followed by streptavidin-peroxidase and OPD. Binding was expressed as a percentage of the maximal OD obtained for 101F MAB with Long virus-infected cell extract.

Microneutralization test. Before being used in the microneutralization test, the stocks of wild-type and mutant viruses were titrated as follows: serial dilutions of viruses in DMEM 2.5 (50 μ l) were used to infect HEp-2 cells growing in 96-well culture plates. After 1 h for adsorption, DMEM 2.5 (up to 150 μ l) was added to each well, and incubation continued for 72 h at 37°C in a 5% CO₂ atmosphere. At this time, medium was removed and, after washing with 0.05% Tween 20 in PBS, cells were fixed with chilled 80% acetone in PBS for 20 min. After air drying, the amount of viral antigen in each well was measured by ELISA with a pool of MAbs specific for hRSV F (2F, 101F, and 56F) and G glycoproteins (021/1G and 021/21G), as previously described (46). The dilution of each virus that yielded 90% of maximal viral antigen was chosen for subsequent microneutralization tests.

The set dilution of each virus stock was incubated with serial dilutions of Nanobodies (Nb017, ALX-0171, or irrelevant IRR26 and IRR33) or MAbs (Pz, 47F, and 101F) for 30 min at room temperature before infection of duplicate cultures of HEp-2 cells growing in 96-well plates (47). The amount of viral antigen produced after 72 h at 37°C was determined by ELISA as indicated in the previous paragraph and expressed as the percentage of the value obtained in cultures infected with each virus in the absence of antibody. Fifty percent inhibitory concentrations (IC₅₀s) were calculated with GraphPad Prism software (version 6.07). The concentration-response curves were fitted with the nonlinear 4PL method.

Selection of escape mutants resistant to Nanobodies. Ten individual plaques of the Long strain of hRSV were isolated from agar plates and grown in HEp-2 cells to obtain clonal virus stocks. Each viral clone was titrated for antigen production in HEp-2 cells as indicated above. The dilution of each virus that yielded 90% of the maximal value was then incubated with serial dilutions of either Nb017 or ALX-0171. The estimated IC₉₀ of these Nanobodies was chosen as the concentration for selection of escape mutants.

Set amounts of virus and Nanobodies were mixed in 50 μ l of DMEM 2.5 and used to infect HEp-2 cell monolayers growing in 96-well plates in a total volume of 150 μ l. After infection, the plates were incubated at 37°C for 4 to 5 days. At this time, visual inspection of the cultures revealed an almost complete absence of cytopathic effect, except in control wells in which viruses were incubated without added Nanobodies. Subsequently, 25 μ l of each culture supernatant was mixed with fresh Nb017 or ALX-0171 Nanobodies and used to infect new cultures. Every five passages, 25 μ l of culture supernatant was used to infect HEp-2 microcultures either in the presence or absence of the selecting Nanobody, and the amount of viral antigen produced after 72 h was measured by ELISA, as described in above.

After 15 passages, viruses present in culture supernatants that were not neutralized by the selecting Nanobody were isolated and allowed to form plaques on agar plates. A single virus plaque from each culture was isolated and grown in HEp-2 cells. Total RNA was isolated from virus-infected HEp-2 cells, and the F gene was amplified and sequenced as described previously (48).

Generation, purification, and characterization of wild-type and mutant forms of the hRSV (Long strain) F glycoprotein. Soluble forms of the wild-type hRSV F glycoprotein (Long strain) folded in either the prefusion or postfusion conformation were obtained as described previously (49). Briefly, recombinant vaccinia viruses were obtained that expressed the ectodomain (amino acids [aa] 1 to 524) of the F protein followed by the foldon trimerization domain (50) and a 6 $\times$ His tag. This protein folded spontaneously in the postfusion conformation (F post) and had a deletion of the first 10 residues of the fusion peptide (aa 137 to 146) and thus avoided aggregation. The F protein folded in the prefusion conformation (F pre) was obtained by incorporating the amino acid changes described by McLellan et al. (51) in their DS-Cav1 mutant (S155C, S190F, V207L, and S290C) to the Long F protein ectodomain, followed by the foldon sequences and the 6 $\times$ His tag. Finally, the changes N262Y (A797U) and S275F (C837U) were introduced in the plasmids harboring genes for the postfusion and prefusion soluble proteins, using the Phusion site-

directed mutagenesis kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Recombinant vaccinia viruses were obtained by the method of Blasco and Moss (52). Proteins were purified from the supernatants of CV-1 cells infected with each recombinant vaccinia virus, using Ni²⁺ columns followed by gel filtration chromatography in a Hi-Load 16/600 Superdex column. Purity and integrity of the different proteins were checked by SDS-PAGE, and their conformation properties were confirmed by electron microscopy and by reactivity in ELISAs with MAb D25, which is specific for the prefusion conformation (33), and MAb 114F, which is specific for the postfusion conformation (53). These ELISAs were performed by capturing serial dilutions of the purified proteins with MAbs bound to 96-well microtiter plates and revealing the amount of protein to each well with an anti-His MAb, as described previously (49).

Surface plasmon resonance. All surface plasmon resonance determinations were done in a Biacore X100 instrument (GE Healthcare). Briefly, an anti-His MAb (GE Healthcare) was amine coupled to a CM5 sensor chip at a density of 12,000 to 13,000 relative units (RU) in 10 mM acetate buffer (pH 4.5). Approximately 100 RU of His-tagged F proteins was bound to the anti-His MAb. The Pz Fab fragment (obtained with the Thermo Scientific kit, catalog number 44985) in PBS–0.05% Tween was injected at a flow rate of 40 μ l/min at the concentrations indicated in the figure legends. Association and dissociation phases were 135 s and 300 s, respectively. Surfaces were regenerated using 30 mM HCl. Data were double-reference subtracted and fitted to a 1:1 Langmuir binding model for the calculation of the kinetic parameters k_{on} and k_{off} . The K_D was then calculated as the ratio of k_{off} and k_{on} .

RESULTS

Multivalent Nanobody formatting enhances neutralization efficiency. As a test reference for those antibodies described in later sections, binding of the Nanobodies and MAbs used in this study to the F protein of wild-type-infected HEp-2 cell extracts (Long strain) was assessed by ELISA (Fig. 1A). Additionally, the potency of the same Nanobodies and MAbs to neutralize the Long virus was evaluated in a microneutralization test (Fig. 1B).

Nanobodies Nb017 and ALX-0171 and MAbs palivizumab, 47F, and 101F all bound to the F protein of Long virus-infected cell extracts (Fig. 1A). It is worth noting that since the detection method and the biotinylation efficiencies may not have been the same for different molecules, no direct comparison could be made between the binding curves of the different compounds shown in Fig. 1A. Furthermore, comparison of binding of biotinylated molecules in a monovalent (Nb017), bivalent (MAbs), or trivalent (ALX-0171) mode is not straightforward. Hence, the binding curves shown in Fig. 1A should not be compared with the reported affinities of Nanobodies and MAbs for prefusion hRSV F (41), since most likely the F protein present in cell extracts used here was not folded in the prefusion conformation (see below). Therefore, Fig. 1A is only shown here as a reference to compare the binding of Nanobodies and MAbs to the F protein of wild-type-infected cell extracts with their binding to F protein mutants, described below.

As reported, Nanobody ALX-0171 neutralized hRSV Long virus about 10,000 times more potently than did monovalent Nb017 (41), whereas MAbs palivizumab, 47F, and 101F neutralized virus infectivity with similar intermediate potencies (Fig. 1B). These results are also shown here as reference to compare the neutralization potency of wild-type virus by each Nanobody or MAb with the neutralization of escape mutants shown, described below.

Isolation of hRSV mutants resistant to monovalent and trivalent Nanobodies. To explore if the enhanced neutralization po-

FIG 1 ELISA binding of Nanobodies and MABs to the F protein of hRSV-infected cell extracts and neutralization of viruses with Nanobodies and MABs. (A) Serial dilutions of the Nanobodies and MABs indicated on the left (symbols) were tested for binding to the F protein present in an extract of hRSV (strain Long)-infected HEp-2 cells, as described in Materials and Methods. The results shown are the percentage of the maximal OD value obtained with MAb 101F on strain Long and are means of duplicates $\pm$ standard deviations. (B) The microneutralization test was performed, as described in Materials and Methods, to measure the amount of viral antigen produced in HEp-2 cells infected for 72 h with hRSV (Long strain) in the presence of serial dilutions of the indicated Nanobody or MAb. The amounts of F and G proteins were estimated by ELISA with a pool of MABs, and the results (mean of duplicates $\pm$ standard deviations) are expressed as the percentage of the OD value obtained in the absence of antibody. Mock-infected cell values were subtracted from these values. IRR26 and IRR33 are monovalent and trivalent irrelevant (negative-control) Nanobodies, respectively.

tency of ALX-0171 compared with that of Nb017 could impact escape mutant selection, the Long virus was serially passed in the presence of suboptimal neutralizing concentrations of the two Nanobodies. To ensure that each mutant originated from independent mutagenic events, 10 independent clones of the Long strain were passed in parallel 15 times in the presence of the indicated Nanobodies. Emergence of viral escape mutants during the selection process was monitored by testing aliquots of culture supernatants every five passages for the presence of Nanobody-resistant viruses in a microneutralization test.

Figure 2 shows that after 5 passages, viruses were found in all culture supernatants and they remained sensitive to Nanobodies Nb017 or ALX-0171, since their infectivity, measured as the amount of viral antigen produced in newly infected cells, was reduced in the presence of the respective Nanobodies. However, after passage 10, viruses present in six of the Nb017-containing cultures (clones 1, 4, 5, 7, 8, and 10) showed increased viral antigen production, measured by ELISA, in the presence of this Nanobody, indicating that escape mutants were apparently already being selected. Nevertheless, passage of the 10 viral clones in the presence of Nb017 was maintained until passage 15. In contrast, viruses passed 10 times in the presence of ALX-0171 were still fully neutralized by this Nanobody. The emergence of viruses resistant to ALX-0171, as shown by lack of neutralization with this Nanobody, was only detected after five more passages (passage 15) in six individual cultures (clones 1, 5, 6, 7, 8, and 10). In other words, selection of escape mutants with ALX-0171 required more passages than with its monovalent counterpart.

At the end of the selection process a single viral plaque was isolated from each culture to avoid mixture of viruses and ensure homogeneity of the mutant stock, and the F protein gene was sequenced, as described previously (48). The amino acid changes in each mutant are shown in Table 1. All changes were clustered between amino acids 258 and 276, which delineate antigenic site II of hRSV F, where changes previously selected with MABs had also been mapped (28). These results were therefore in full agreement with the previously reported lack of binding of Nb017 and ALX-

0171 to mutants of antigenic site II, whereas their binding was unaltered for mutants with altered antigenic site IV of hRSV F (41). Some mutations were selected only with the monovalent Nb017 Nanobody (e.g., N262I), whereas others were selected with both the mono- and the trivalent Nanobody (e.g., N276Y), although the number of mutants analyzed was too small to draw any firm conclusions about the influence of Nanobody valence in selected mutations. Notably, mutations in residues 262 and 276 were particularly favored among the escape mutants selected with the Nanobodies (8 out of 12). While some of the mutations in Table 1 had been already isolated with certain MABs, others were found only in escape mutants selected with the Nanobodies (e.g., N262I and S275T). In contrast, mutations in residue 272 selected with MAb 7C2 (44) were not found among the mutants selected with the Nanobodies (Table 1).

Binding and neutralization of escape mutants with Nanobodies and MABs. In order to assess the influences of mutations selected in escape mutants on binding and neutralization by Nanobodies, a subset of the mutants shown in Table 1 that bore unique amino acid substitutions were chosen to infect HEp-2 cells. Extracts of these infected cells were tested in ELISAs for binding to the noted Nanobodies. In addition, the MABs shown in Fig. 1 that bind epitopes of either site II (47F and Pz) or site IV (101F) of hRSV F were included in the study for comparison. In parallel, the neutralizing potencies of Nanobodies and MABs with either Long or escape mutants were assessed in an *in vitro* microneutralization test.

To broaden the mutations of antigenic site II of hRSV F that were being evaluated, two escape mutants (R7C2/1 and R7C2/11) previously selected with MAb 7C2 (44) and that contained changes (K272E or K272T) not present in any of the Nb017- or ALX-0171-selected mutants, were included in the study. The ELISA and neutralization results obtained with each mutant virus are shown in Fig. 3. They should be compared with those of Fig. 1, obtained with wild-type hRSV (Long strain). Although the irrelevant Nanobody controls were included in all tests, these data have been removed from Fig. 3 for clarity.

FIG 2 Evaluation of infectivity after different passage numbers for the escape mutant selection process. Ten individual clones of the hRSV Long strain (abscissae) were passed repeatedly in the presence of either Nb017 or ALX-0171, as described in Materials and Methods. At passages 5, 10, and 15, 25 μ l of culture supernatant was used to infect HEP-2 cells in the absence or presence of the corresponding Nanobody. The amount of viral antigen produced in each case was estimated by ELISA as for the microneutralization assay shown in Fig. 1. Note that virus was lost in some clones (e.g., clone 3 of the Nb017 series), others remained susceptible to neutralization throughout the selection process (e.g., clone 3 in the ALX-0171 series), and finally, other clones showed the presence of escape mutants after 10 or 15 passages (e.g., clone 5 in the Nb017 series).

The results shown in Fig. 3 can be summarized in five points, as follows. (i) Each mutant showed a unique profile of binding and neutralization with Nanobodies and MAbs. Most mutations altered binding and/or neutralization with both Nanobodies and

MAbs of antigenic site II (47F and Pz), supporting the idea that they all recognized overlapping epitopes. In contrast, binding and neutralization with MAb 101F (site IV) were unaffected by any of the mutations, as expected.

TABLE 1 Escape mutants selected with Nanobodies

Nanobody used for selection	Virus ^a clone	Mutant name	Nucleotide change	Amino acid change	Other Abs that selected same mutation ^b
Nb017	1	RNb017/2/1	A816U	N268I	47F, 11, Pz, poly anti-F
	4	RNb017/2/13	U836A	S275T	
	5	RNb017/2/14	A839U	N276Y	
	7	RNb017/2/17	A798U	N262I	Pz
	8	RNb017/2/18	A798U	N262I	Pz
	10	RNb017/2/21	A839U	N276Y	
ALX-0171	1	RALX/1	A839U	N276Y	Pz
	5	RALX/14	A797U	N262Y	47F, AK13, Pz
	6	RALX/16	A797U/A1147G	N262Y/no change	47F, AK13, Pz
	7	RALX/17	C837U	S275F	1129
	8	RALX/18	A797U	N262Y	47F, AK13, Pz
	10	RALX/21	U786C	L258S	7C2

^a Virus clone numbers refer to those in Fig. 2.

^b Other antibodies that selected the same mutations as those selected by the Nanobodies. The following publications described the mutants selected with MAbs 7C2, AK13, or 11 (44), Pz (23, 24, 56), 1129 (57), and polyclonal rabbit anti-F antiserum (poly anti-F) (48).

FIG 3 ELISA and neutralization test results for escape mutants by using Nanobodies and MAb. The results should be compared with those of wild-type virus (Fig. 1) as reference. ELISA and neutralization test ordinate values were also calculated as for Fig. 1. IRR26 and IRR33 negative controls were included in all assays, but they have been omitted from the graphs for clarity.

(ii) Certain mutations, such as N262I or N276Y, abrogated or greatly reduced neutralization by Nb017 or ALX-0171 but had minimal effects on neutralization by MAbs 47F and Pz. In contrast to the findings with the mutations N262I or N276Y, other mutations, such as K272T, which abrogated neutralization by MAbs 47F and Pz, had only minor effects on neutralization by the two Nanobodies. Finally, certain mutations, such as N262Y and S275F, differentially altered binding and neutralization by the two otherwise-similar MAbs 47F and Pz.

(iii) The N268I mutation, which is frequently selected with monoclonal and polyclonal antibodies (48), had a minimal im-

pact on neutralization by Nb017 and ALX-0171, despite being selected in this study with Nb017.

(iv) In general, loss of neutralization with Nb017 was found more frequently among escape mutants than with ALX-0171; i.e., neutralization with the trivalent Nanobody was more difficult to abrogate by mutation than neutralization with the monovalent Nanobody, in line with the difficulty of selecting escape mutants with ALX-0171.

(v) Generally, mutations that reduced ELISA binding also prevented neutralization by a given Nanobody or MAb. However, there were certain exceptions. Most notably, the N262Y mutation

only moderately decreased Pz binding, yet the mutant virus was fully resistant to Pz neutralization. On the contrary, S275F very significantly reduced 47F binding but had only a modest effect on neutralization.

It is likely that as reported previously (32) the F protein present in the cell extracts was folded in the postfusion conformation. Although both 47F and Pz epitopes are shared by the prefusion and postfusion conformations of hRSV F, the possibility existed that they were differentially modified by the N262Y and S275F mutations in the two F protein conformations, thus explaining the conflicting effects on antibody binding and neutralization observed in Fig. 3. To test this hypothesis, soluble forms of wild-type and mutant hRSV F proteins, stabilized in either the prefusion or postfusion conformation, were produced and tested for ELISA binding with conformation-specific MAbs and with 47F and Pz (Fig. 4).

First, the reactivity of the different proteins with 101F (Fig. 4A), whose epitope is shared by the prefusion and postfusion conformations of hRSV F, confirmed that the amount of each protein in the assays was essentially the same (32). Then, the reactivity of the different proteins with the prefusion-specific MAb D25 (Fig. 4B) and the postfusion-specific MAb 114F (Fig. 4C) confirmed that all proteins were folded in the expected conformation. More meaningfully, however, were the binding results with the different proteins to MAbs 47F (Fig. 4D) and Pz (Fig. 4E).

Whereas MAb 47F recognized the prefusion and postfusion wild-type F proteins with similar efficiency, binding to the prefusion N262Y mutant was reduced about 100-fold, concurring with the reduced neutralization of this mutant by MAb 47F (Fig. 3), although binding to the postfusion N262Y mutant was only moderately reduced, in agreement with the results shown in Fig. 3. Binding of MAb 47F to the postfusion S275F mutant was reduced about 50-fold with respect to the wild-type protein (Fig. 4D), a result compatible with the reduced binding of this MAb observed in the experiment summarized in Fig. 3, but binding of 47F to the prefusion S275F was essentially unchanged (Fig. 4), in agreement with the efficient neutralization of this mutant by MAb 47F (Fig. 3).

The conflicting results of binding and neutralization noted with the MAb Pz (Fig. 3) were also reflected in the results summarized in Fig. 4E. Thus, whereas the prefusion and postfusion forms of wild-type F were equally recognized by Pz, the same two forms of mutant S275F failed to bind to this antibody, in agreement with the reduced binding and lack of neutralization observed in Fig. 3. Binding of Pz to postfusion N262Y was substantially reduced (Fig. 4E), reflecting the reduced binding observed in Fig. 3, but the binding of Pz to prefusion N262Y was essentially abrogated (Fig. 4), compatible with the absence of neutralization of this mutant by Pz (Fig. 3).

To rule out possible ELISA artifacts, binding of the Pz Fab fragment to the wild-type prefusion and postfusion forms of hRSV F as well as to the N262Y mutant proteins was assessed by surface plasmon resonance (Fig. 4F to J). Wild-type postfusion F showed a slightly higher affinity than prefusion F for Pz Fab, and this was reflected in an 8-fold difference of the calculated K_D values (Fig. 4J). However, binding of Pz Fab to the mutant proteins was so low that calculation of the kinetic constants was not feasible. Nevertheless, binding of Pz Fab to the postfusion N262Y mutant was somewhat higher than to the prefusion mutant, reflecting the ELISA binding results (Fig. 4E).

In summary, the results shown in Fig. 3 and 4 highlight the complexities of hRSV neutralization, which correlates better with binding to prefusion F than to postfusion F, as expected from the mode of action described for neutralizing antibodies (46).

The effects of different mutations on neutralization by Nanobodies and MAbs are summarized in Table 2, with the estimated IC_{50} of each Nanobody or MAb relative to MAb 101F. MAb 101F was chosen as the reference because its epitope was not disrupted by any of the mutations shown in Table 2. As noted, the IC_{50} calculated for Nb017 with wild-type virus (Long strain) was 123.3-fold higher than that for 101F, whereas the IC_{50} calculated for ALX-0171 was 200-fold lower than that for 101F. Furthermore, viruses with mutations that prevented neutralization by Nb017, such as L258S, were still neutralized by ALX-0171 at concentrations lower than those of 101F. It should also be noted that viruses fully resistant to Pz, such as R7C2/1 or R7C2/11 (Table 2), were still neutralized by ALX-0171 at concentrations 5 or 10 times lower than those of 101F. Indeed, only two mutants (N262I and S275F) were fully resistant to ALX-0171 neutralization. This enhanced neutralizing potency of ALX-0171 might explain the increased difficulty in isolation of escape mutants, in comparison with Nb017.

To visualize the mutations that more drastically altered neutralization by the Nanobodies and MAbs shown in Table 2, the shift of the IC_{50} versus that with the Long strain for each mutant virus was calculated; values >30-fold different are shaded in Table 2. Although the range of mutations that increased the IC_{50} overlapped in Table 2 mutants, each Nanobody or MAb showed a unique pattern of sensitivity to different mutations. Nb017 and ALX-0171 were drastically altered by the mutations N262I, N262Y, K272E, S275F, S275T, and N276Y, but L258S had much less of an effect on ALX-0171 than on Nb017. Similarly, most of the mutations that drastically altered neutralization by MAbs 47F and Pz were the same, but S275F had a much lower effect on 47F than on Pz. These results highlight the individuality of each Nanobody or MAb despite recognizing highly overlapping epitopes.

Tables 1 and 2 provide an extensive catalog of mutations that alter epitopes of the hRSV F antigenic site II that have been reported to date. In some cases, the same mutations were selected in viruses resistant to Nanobodies or MAbs, but in other cases certain mutations were only found in mutants selected with Nanobodies (e.g., N262I and S275T).

DISCUSSION

One of the main concerns for the clinical use of antivirals is the emergence of escape mutants that could make treatments ineffective. In the case of hRSV, escape mutants which resist Pz prophylaxis in cotton rats could readily be isolated *in vitro* (54), although some of these mutants showed reduced fitness in comparison with wild-type virus (23). Furthermore, mutations associated with Pz resistance were only found in about 5% of clinical samples from infants that were diagnosed with lower respiratory tract RSV disease and that had received prophylactic treatment with that MAb in a clinical trial (24). This frequency of resistant viruses was not considered of major concern, since the prophylactic use of Pz is limited to children at high risk of severe hRSV infections, which represents only a minor fraction of the susceptible population to hRSV. How-

FIG 4 Binding of wild-type and mutant forms of hRSV to MAbs. (A to E) The MAbs indicated in each panel were used to coat 96-well microtiter ELISA plates. Then, serial dilutions of the indicated proteins were added, and ELISA binding was assessed as described in Materials and Methods. (F to J) Surface plasmon resonance sensorgrams for Pz Fab binding to the proteins indicated in each panel. Data were double-reference subtracted and fit to a 1:1 binding model (red lines) with the wild-type prefusion and postfusion proteins for calculation of kinetics constants (J). Poor binding of Pz Fab to the N262Y mutant proteins precluded fitting of sensorgrams and determination of kinetics constants. Note, however, that binding of Pz Fab to postfusion the N262Y mutant was somewhat more efficient than to the mutant prefusion protein, as seen in the ELISA in panel E.

ever, were Pz used more widely, the emergence of resistant viruses might be an issue of concern.

Nanobodies have shown promising results as inhibitors of hRSV replication *in vitro* (37, 41) and in animal models (39, 41). Indeed, multivalent and multiparatopic Nanobodies compared

favorably with Pz in *in vitro* neutralization tests (37, 41). However, no information was yet available about either the capacity of Nanobodies to select escape mutants or the type of mutants that could be selected. The results of Fig. 3 indicate that single point mutations in the F protein are sufficient to confer Nanobody re-

TABLE 2 Neutralization indices for Nanobodies and MAbs against wild-type and escape mutant viruses analyzed in this study

Virus strain ^a	Ab used for selection	Amino acid change	Neutralization indices (fold difference in IC ₅₀ s) for Nanobody or MAb ^b									
			Compared to MAb 101F					Compared to response of strain Long				
			Nb 017	ALX-0171	47F	Pz	101F	Nb 017	ALX-0171	47F	Pz	101F
Long		Wild type	101.6	0.005	2.0	1.7	1.0	1.0	1.0	1.0	1.0	1.0
RALX/21	ALX-0171	L258S	10,985	0.067	2.8	2.1	1.0	108.1	14.5	1.4	1.2	1.0
RNb017/2/18	Nb017	N262I	>>	>>	0.4	0.2	1.0	>>	>>	0.2	0.1	1.0
RALX/14	ALX-0171	N262Y	55,462	1.1	2,756	2,187	1.0	546.0	241.1	1,368	1,284	1.0
RNb017/2/1	Nb017	N268I	138.8	0.006	97.6	71.0	1.0	1.4	1.3	48.4	41.7	1.0
R7C2/1	MAb 7C2	K272E	9876	0.2	424.7	>>	1.0	97.2	46.6	210.8	>>	1.0
R7C2/11	MAb 7C2	K272T	707.3	0.1	224.2	426.8	1.0	7.0	22.2	111.3	250.7	1.0
RALX/17	ALX-0171	S275F	>>	>>	25.9	>>	1.0	>>	>>	12.8	>>	1.0
RNb017/2/13	Nb017	S275T	3167.1	0.4	3.7	2.0	1.0	31.2	91.6	1.8	1.2	1.0
RALX/1	ALX-0171	N276Y	>>	7.2	4.6	3.6	1.0	>>	1,554	2.3	2.1	1.0

^a Mutants selected with MAb 7C2 (shown in boldface) were described by Arbiza et al. (44) and are included here for comparison with other mutants, since their particular amino acid changes were not selected with the Nanobodies we used.

^b The IC₅₀s relative to those for MAb 101F were calculated from the neutralization test results summarized in Fig. 3. We divided the amount of each Nanobody or MAb that reduced 50% antigen production by the amount of MAb 101F that had the same effect. Then, IC₅₀s relative to strain Long were calculated by dividing the IC₅₀ of each virus with each Nanobody or MAb by that of the Long strain with the same Nanobody or MAb. >>, the IC₅₀ was unresolved for the virus strain. Shaded values for Nanobodies and MAbs with IC₅₀s >30-fold above that for Long or for which the IC₅₀ was unresolved highlight the mutations depicted in Fig. 5.

sistance. By analogy to Pz escape mutants that incorporated amino acid changes at F protein residues involved in direct interactions with that antibody (30), it is likely that the amino acid changes conferring resistance to Nanobodies are also present in residues directly involved in Nanobody binding. In other words, neither long distance nor allosteric effects seem to play a role in Nanobody resistance.

Site II is mainly determined by a helix-loop-helix motif (55) that is preserved in both the prefusion and the postfusion conformations of hRSV F (33). Based on the structure of a synthetic peptide spanning residues 255 to 276 of the F protein bound to motavizumab Fab (Mz, an optimized version of Pz) (30), McLellan et al. modeled the complex of Mz bound to postfusion F (31). Their model showed that most residues in the two α-helices, but not in the loop, interacted with residues of the heavy and light

chains of Pz, in full agreement with the location of escape mutations selected in Pz-resistant mutants (Fig. 5).

As noted, the mutations selected with Nb017 or ALX-0171 overlapped with those selected with MAbs 47F and Pz (Fig. 5). However, the mutations L258S and N276Y, which have major effects on neutralization by Nanobodies but not by MAbs (Table 2), are very close to the core of the F protein, suggesting that Nanobodies may reach residues which are embedded in the F protein and that are not accessible to larger molecules, such as MAbs. These binding requirements may also explain the differences in the neutralization profiles with escape mutants between Nanobodies and MAbs (Table 2).

The procedure used in this study for the isolation of escape mutants included multiple passages of the Long virus in the presence of suboptimal amounts of Nanobodies. These conditions

FIG 5 Location of escape mutant amino acid changes on the prefusion hRSV F structure. The three-dimensional structure of the prefusion hRSV F trimer (left) was built with the coordinates provided by McLellan et al. (50) (PDB ID 4MMT), using PyMol software. One of the protomers is displayed as a gray ribbon. The antigenic site II motif, identified by McLellan et al. (29), is highlighted in green. The surfaces of the other two protomers are represented in pale pink and gold. The oval area of antigenic site II was enlarged and copied four times to indicate in each copy the mutations that increased the IC₅₀ over strain Long >30-fold for the indicated Nanobodies and MAbs (Table 2).

may reflect to a certain extent the prophylactic situation found *in vivo* where it is unlikely that the incoming virus will be faced with a large excess of antibody. However, in a therapeutic setting, where the Nanobody is delivered directly to the lungs, a large excess of antibody is expected (40).

Since multiple passages were required for the emergence of mutants, it was impossible to estimate the frequency of mutations generated during each round of virus replication. Nevertheless, the number of passages at which mutants resistant to Nb017 started to emerge (between passages 5 and 10) was comparable to those previously seen in our laboratory for the isolation of mutants resistant to MAbs of antigenic site II (44) or IV (28). This is in contrast with the situation found with the trivalent ALX-0171, which required more passages for the emergence of resistant viruses.

In summary, the most significant findings of this study are (i) the increased difficulty to isolate mutants resistant to the trivalent Nanobody compared with the monovalent Nanobody and (ii) the increased capacity of ALX-0171 to neutralize escape mutants (even those selected with this Nanobody), compared with Nb017 and MAbs. Enhanced neutralizing potency was previously reported for other bivalent and biparatopic Nanobodies (37), but nothing was known about the behavior of monovalent or trivalent Nanobodies in neutralization of escape mutants. The basis for the neutralizing enhancement observed with ALX-0171 is not known, but it is worth mentioning that this increase in neutralization potency correlated with an increase of about 160-fold in binding affinity for prefusion F (41) in comparison with Nb017. In any case, the high neutralizing potency of ALX-0171, its decreased capability to select escape mutants, and its increased capacity to neutralize the noted mutants are characteristics which make this Nanobody an attractive hRSV-specific antiviral.

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