CLINICAL IMPLICATIONS OF BASIC RESEARCH

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Preparing for the Future — Nanobodies for Covid-19?

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Many had hoped that monoclonal antibody drugs would provide an important stopgap to the coronavirus disease 2019 (Covid-19) pandemic by limiting severe disease and thus the number of hospitalizations until safe and effective vaccines could be approved.[1] Despite the emergency use authorization issued by the Food and Drug Administration (FDA) for antibody drugs on the basis of their ability to reduce viremia in mildly and moderately ill patients with Covid-19, only a small proportion of the nation's supply has been used. Myriad challenges include the therapeutic window (these drugs are more effective when administered during the first 4 to 7 days in the course of illness), the sheer number of patients during a pandemic surge and the relative paucity of infusion centers and medical staff professionals, and the emergence of mutations that affect the spike protein, which could lead to increased transmissibility and the potential for resistance to neutralization by antibodies.[2] Therefore, new therapies that are effective against variants and offer an alternative to intravenously administered antibody drugs are highly desired.

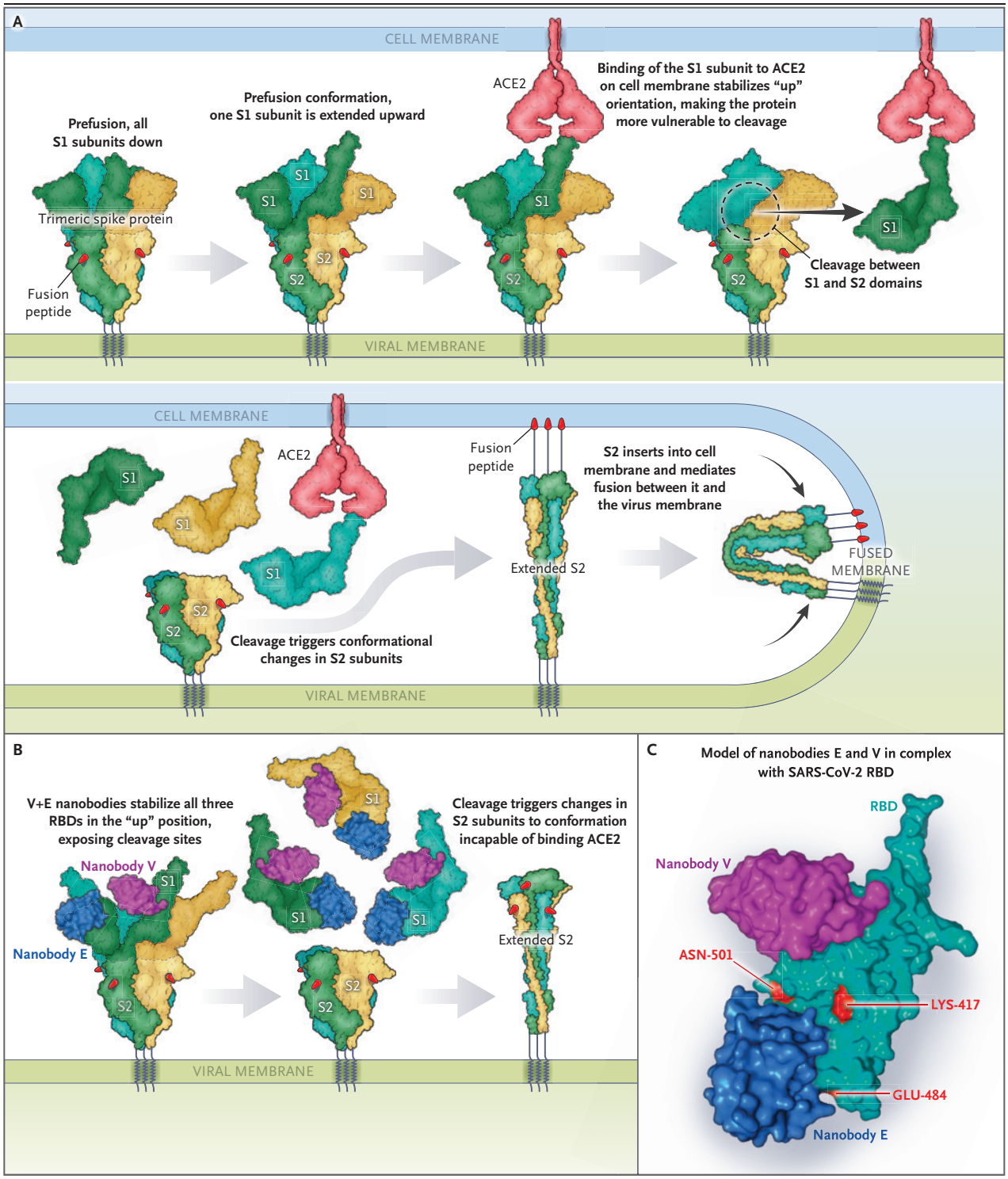
A study by Koenig and colleagues[3] on camelid-derived, single-domain antibodies (or nanobodies) is therefore timely. The researchers immunized alpacas and llamas with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike protein and identified nanobodies that specifically bind to the receptor-binding domain of the virus. They characterized four neutralizing nanobodies (labeled E, U, V, and W) structurally and functionally with multiple in vitro assays. Three of the nanobodies (U, V, and W) recognize a common epitope located near the threefold axis of the prefusion trimeric spike, whereas nanobody E recognizes the extended loop (residues R466 through P491) overlapping the receptor-binding domain (Fig. 1C). The nanobodies bound the receptor-binding domain of the virus with an equilibrium dissociation constant of between 2 and 22 nmol and neutralized SARS-CoV-2 infection by 50% in a plaque-reduction assay at concentrations ranging from 48 to 185 nmol, results similar to those achieved with monoclonal antibodies.[5] In contrast to the V nanobody, nanobodies E, U, and W have the potential to prevent SARS-CoV-2 from binding angiotensin-converting enzyme 2 (ACE2) on host cells, in agreement with the location of the epitopes to

Figure 1 (facing page). SARS-CoV-2 Membrane Fusion Process and Footprints of V and E Nanobodies.

The spike protein of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is made up of subunit 1 (S1) and subunit 2 (S2). Trimers of the spike protein are expressed on the surface of the virus (Panel A). In the prefusion conformation, one S1 subunit is extended upward. Binding of the S1 subunit to angiotensin-converting enzyme 2 (ACE2) on the cell membrane stabilizes the "up" orientation, which is likely to induce proteolytic cleavage. Cleavage triggers conformational changes in S2 subunits. This reconformation involves the extension of the fusion peptide into the host-cell membrane, whereupon S2 draws together the host-cell membrane and the viral membrane. Koenig et al.[3] recently reported that V+E nanobodies stabilize all three RBDs in the "up" position. This stabilization likely permits proteolytic cleavage and premature structural transition, without leading to membrane fusion (Panel B). Nanobodies V and E are shown in complex with the RBD of SARS-CoV-2 (Protein Data Bank codes 7KSG and 7KN6) (Panel C). Residues modified in the B.1.1.7 and B.1.135 variants (K417, E484, and N501) are colored red. The average network score for nanobody V and E epitopes are 0.344 and 0.326, respectively. The average network score for the ACE2 structural epitope, which overlaps with the footprint of E, is 0.325. The network score is a measure of structural constraints on a residue resulting from interatomic interactions; higher scores indicate more constraints, and lower scores indicate increased susceptibility to undergo mutation.[4]

which they bind and their mode of engagement with the receptor-binding domain. The nanobodies neutralize the virus by inducing a premature structural transition from a prefusion conformation to an irreversible postfusion conformation, the latter of which is incapable of binding ACE2 and thus incapable of triggering membrane fusion.

The authors then made biparatopic nanobodies



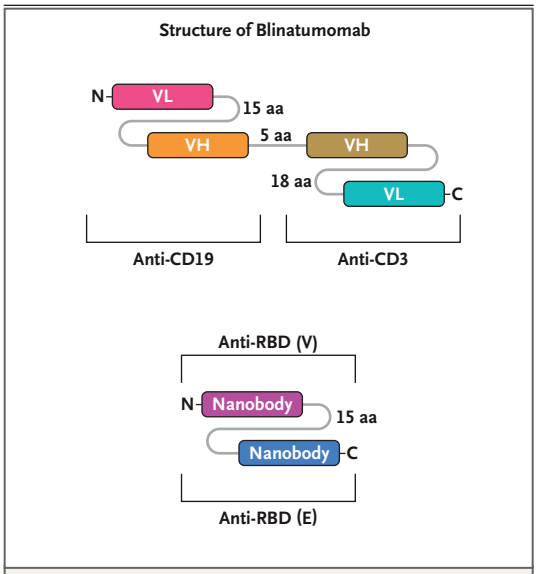


Figure 2. Modular Organization of Bispecific Blinatumomab and a Biparatopic SARS-CoV-2 Nanobody.

Shown is the organization of bispecific blinatumomab (upper diagram) and the biparatopic SARS-CoV-2 nanobody V+E (lower diagram). In blinatumomab, the single-chain variable fragment of the anti-CD19 antibody is linked to the single-chain variable fragment of anti-CD3 antibody by a short linker of five amino acids (upper diagram). The structure of biparatopic SARS-CoV-2 nanobodies is similar to that of a single-chain variable fragment: it is formed by the two camelid single-domain (heavy chain) antibodies (i.e., two nanobodies) connected by a linker of 15 amino acids (lower diagram). Both antibodies lack the crystallizable Fc region. VH denotes variable heavy and VL variable light.

(i.e., nanobodies that have two antigen-binding sites in one molecule) by fusing nanobodies that targeted distinct epitope regions (e.g., E+V, V+E, E+W, and W+E). Using cryoelectron microscopy, they showed that the most potent biparatopic nanobody (V+E) binds to all three spike proteins of the trimer (nanobody-to-trimer, 1:3 stoichiometry) with all the receptor-binding domains in the "up" conformation, indicating that the binding of nanobodies stabilizes the receptor-binding domain and prevents up–down motion, most likely contributing to proteolytic cleavage of the spike and premature transition to an irreversible postfusion conformation. The V+E biparatopic nanobody neutralized SARS-CoV-2 infection at a dilution 62 times greater than that achieved by the individual nanobodies, possibly because of the improved avidity to the spike protein (an affinity that is at least 22 times greater than that of individual nanobodies).[4]

While passaging a chimeric virus in Vero E6 cells in the presence of nanobodies E, U, V, and W, but not in the presence of the biparatopic (V+E or E+V) nanobodies, the authors found escape variants that had mutations within the epitope regions. This observation highlights the advantage of simultaneously targeting more than one vulnerable epitope. Of note, the footprint of the V nanobody does not include amino acids 417, 484, and 501 of the spike protein (Fig. 1C), which are changed in the strains recently identified in Britain, South Africa, and Brazil, suggesting that the biparatope antibody V+E (or E+V) would be effective against these antigenic variants. The epitope recognized by nanobody V is relatively more constrained than the E epitope (which includes residues E484 and N501), meaning it is less likely to tolerate changes caused by mutation. Therefore, mutations that arise in the part of the S gene that encodes this region (i.e., the region of the spike to which the V nanobody binds) are less likely to survive selection.

Koenig et al. have contributed to the growing number of studies that have isolated nanobodies against SARS-CoV-2. Owing to the relatively small size of nanobodies, they have favorable biophysical properties and are cheaper to produce than standard monoclonal antibodies. Their small size and their long, heavy-chain complementarity-determining regions enable them to target concave epitopes such as the receptor-binding site of the spike protein. Nanobodies can be made with the use of prokaryotic expression systems (e.g., from bacteria or yeast) because they lack the glycan-harboring Fc domain, making them easier to manufacture than standard monoclonal antibodies. The absence of an Fc region eliminates the risk of antibody-dependent enhancement of infection, but it also shortens the half-life, which could plausibly be addressed through attachment to or amalgamation with polyethylene glycol or human serum albumin. Moreover, nanobodies can be nebulized and delivered straight to the lungs of a patient with Covid-19 with an inhaler, thus presenting a better logistic alternative to intravenously administered antibodies. Aerosol formulation of nanobodies has shown promising nonclinical results.

Although nanobodies are under clinical in-

vestigation for use in a wide range of diseases from cancer to infectious diseases, it was the approval of caplacizumab (an anti–von Willebrand factor bivalent nanobody) by the European Medicines Agency and the FDA for the treatment of thrombotic thrombocytopenic purpura and thrombosis that marked the foray of nanobodies into clinical medicine. The format of the biparatopic nanobody V+E engineered by Koenig et al., although distinct from that of a conventional nanobody, is similar to that of the FDA-approved single-chain, variable fragment–based bispecific antibody blinatumomab (Fig. 2). All things considered, the available structural and clinical data suggest that the biparatopic antibody could potentially offer a better alternative to conventional monoclonal antibodies for the treatment of Covid-19. Recently, experts representing various organizations including regulatory bodies, academia, and pharmaceutical and biotechnology companies have made a call to develop small-molecule drugs that inhibit the machinery that the virus uses to replicate. Such agents are convenient to administer and insensitive to viral mutations. The biparatopic antibody, when formulated for aerosol or subcutaneous administration, will lend those benefits just as effectively.

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1. Renn A, Fu Y, Hu X, Hall MD, Simeonov A. Fruitful neutralizing antibody pipeline brings hope to defeat SARS-CoV-2. Trends Pharmacol Sci 2020;41:815-29.
2. Andreano E, Piccini G, Licastro D, et al. SARS-CoV-2 escape *in vitro* from a highly neutralizing COVID-19 convalescent plasma. December 28, 2020 (https://www.biorxiv.org/content/10.1101/2020.12.28.424451v1). preprint.
3. Koenig PA, Das H, Liu H, et al. Structure-guided multivalent nanobodies block SARS-CoV-2 infection and suppress mutational escape. Science 2021;371:eabe6230.
4. Soundararajan V, Zheng S, Patel N, et al. Networks link antigenic and receptor-binding sites of influenza hemagglutinin: mechanistic insight into fitter strain propagation. Sci Rep 2011;1:200.
5. Xiang Y, Nambulli S, Xiao Z, et al. Versatile and multivalent nanobodies efficiently neutralize SARS-CoV-2. Science 2020;370:1479-84.

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