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Antibody-mediated synergy and interference in the neutralization of SARS-CoV at an epitope cluster on the spike protein

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Incomplete neutralization of virus, especially when it occurs in the presence of excess neutralizing antibody, represents a biological phenomenon that impacts greatly on antibody-mediated immune prophylaxis of viral infection and on successful vaccine design. To understand the mechanism by which a virus escapes from antibody-mediated neutralization, we have investigated the interactions of non-neutralizing and neutralizing antibodies at an epitope cluster on the spike protein of severe acute respiratory syndrome coronavirus (SARS-CoV). The epitope cluster was mapped at the C-terminus of the spike protein; it consists of structurally intertwined epitopes recognized by two neutralizing monoclonal antibodies (mAbs), 341C and 540C, and a non-neutralizing mAb, 240C. While mAb 341C binds to a mostly linear epitope composed of residues 507PAT509 and V349, mAb 240C binds to an epitope that partially overlaps the former by at least two residues (P507 and A508). The epitope corresponding to mAb 540C is a conformational one, involving residues L504 and N505. In neutralization assays, non-neutralizing 240C disrupted virus neutralization by mAbs 341C and/or mAb 540C, whereas a combination of mAbs 341C and 540C, blocked virus infectivity synergistically. These findings indicate that the epitope cluster on the spike protein may serve as an evolutionarily conserved platform at which a dynamic interplay between neutralizing and non-neutralizing antibodies occurs, thereby determining the outcome of SARS-CoV infection.

Introduction

Patients suffering from infection with severe acute respiratory syndrome coronavirus (SARS-CoV) [1] often have appreciable levels of virus-specific antibody [2–5]. This situation is similar to that encountered in other viral diseases such as chronic hepatitis C or HIV, where the infection persists despite the presence of antibodies [6–10]. One of the mechanisms proposed originally by Dulbecco et al. [11] to account for apparent inhibition of virus neutralization was that the serum of infected individuals might contain non-neutralizing antibodies. When combined with the corresponding virus, the non-neutralizing antibody could presumably interfere with the attachment of neutralizing antibody, thereby diminishing its neutralizing activity. Massey and Schochetman subsequently showed that non-neutralizing antibody binds to virus and sterically blocks the binding of neutralizing antibodies [12]. Recently, we found two Epitopes I and II, within a short peptide between the hypervariable regions I and II of the hepatitis C virus E2 protein. Epitope I, but not Epitope II, was implicated in virus neutralization. The binding of a non-neutralizing antibody to Epitope II disrupted virus neutralization mediated by antibody binding at Epitope I [9,13].

Here, we have investigated whether or not a similar occurrence, namely the presence of non-neutralizing antibody along with neutralizing antibody, could account for the observation that SARS-CoV survives despite a vigorous antibody response by the host. We have characterized three previously isolated monoclonal antibodies (mAbs) that recognize the spike protein of SARS-CoV between residues 491 and 510 [14]. We found that the two neutralizing mAbs, 341C and 540C, could act synergistically to inhibit SARS-CoV infection in vitro, while the non-neutralizing antibody, 240C, disrupted the neutralizing activity of both 341C and 540C. These findings suggest that this epitope cluster may provide a viral escape mechanism whereby the neutralization of virus is thwarted by an interfering, non-neutralizing antibody.

Materials and methods

Monoclonal antibodies. Monoclonal anti-SARS-CoV antibodies, 240C, 341C and 540C, were obtained from the Bedefense and Emerging Infections Research Resources Repository, NIAID, NIH. mAbs 341C and 534C could neutralize SARS-CoV infection of Vero
E6 cells, while mAb 240C did not. The epitopes of these mAbs were located within residues 491–510 on the spike protein. The mAb 540C used in the present study is similar to 534C as described previously [14].

Virus stock and micro-neutralization assay. Vero E6 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine. The Urbani strain of SARS-CoV was plaque-purified, grown to stock titer in Vero E6 cells, purified by polyethylene glycol (PEG) precipitation as described previously [15], and frozen at −70 °C until use.

A micro-neutralization assay was performed as previously described [16]. The micro-neutralization titer of test antibody was the highest dilution that showed inhibition in all triplicate wells. Controls were included for each assay performed and included back titration, inclusion of positive control antibody (i.e., serum from a convalescent SARS patient) and an isotype monoclonal antibody control. Data obtained from at least three independent experiments were analyzed.

Peptide synthesis. All peptides were synthesized by the Core Laboratory of the Center for Biologics Evaluation and Research, Food and Drug Administration, with an Applied Biosystems (Foster City, CA) Model 433A Peptide Synthesizer by using standard FastMoc chemistry [17].

ELISA. Streptavidin-coated 96-well plates were used for ELISA according to the manufacturer's instructions (Pierce, Rockford, IL). Briefly, biotinylated peptides (200 ng/well) were added to streptavidin-coated wells and blocked with Blocking Buffer for 1 h at 37 °C. After washings with PBS with 0.05% Tween 20 (PBS-T), primary antibody was added to the wells and incubated for 45 min at 37 °C. After removal of unbound antibodies by washing with PBS-T, a goat anti-mouse peroxidase-conjugated IgG (KPL, Gaithersburg, MD) at 1:5000 dilution was added to the wells and incubated for 30 min at 37 °C. After washings, tetramethylbenzidine substrate (Pierce) was added and the plates were incubated at room temperature in the dark for 10 min. The reaction was terminated by adding 4 N sulfuric acid, and absorbance at 450 nm was measured (Optimax: Molecular Devices, Palo Alto, CA).

Phage display. Selection of peptides from random peptide phage display libraries (New England Biolabs, Beverly, MA) was described previously [18]. Briefly, 10^{10} phages were incubated with individual antibody/protein G mixtures for 20 min at room temperature. After eight washings with 0.05 M Tris–HCl buffer (pH 7.5) containing 0.15 M NaCl and 0.05% Tween 20, the phages were eluted from the complex with 0.1 M HCl for 8 min at room temperature and neutralized with 1 M Tris–HCl (pH 9.0). The eluted phages were then amplified in the host strain ER2738. After three additional rounds of selection of amplified phages, DNA from each single-phage plaque was sequenced, and the corresponding peptide sequence was then deduced from the DNA sequence.

Structural analysis. The structural analysis was performed by using the coordinates of the SARS-CoV receptor-binding domains (RBD), i.e., PDB IDs 2DD8, 2AJF, 2GHW and 2GHV [19–22]. Among these structures, PDB ID 2DD8 has a complete assignment for the C-terminal region of RBD encompassing residues 491–510 for the antibody binding. The structure was visualized and manipulated by using the program PyMOL 0.99rc6 [23].

Statistical analysis. JMP v.5.0 software (SAS Institute, Cary, NC) was used for analyzing data. Pair-wise comparisons of the means between peptide mutations were performed by using the Student’s t-test where p < 0.05 was considered statistically significant. For an overall comparison of means, the Tukey–Kramer HSD test was used. Statistical significance was set at α = 0.05. A positive test value generated between two means is indicative of a significant difference.

Results

Identification of a cluster of epitopes recognized by mAbs 240C, 341C and 540C at the C-terminus of the spike protein

We attempted to identify the residues within fragment 491–510 that are critical for antibody recognition. A peptide encompassing residues 491–510 was chemically synthesized. ELISA analysis showed that only mAb 341C was capable of recognizing the synthetic peptide, whereas mAbs 240C and 540C were unable to bind to it (Fig. 1A). These results suggested that the epitope corresponding to mAb 341C was likely to be a linear epitope, consisting of residues within peptide 491–510.

To characterize the epitope for mAb 341C antibody, we made use of previously published structural information about the spike protein [19] to guide the synthesis of a set of peptides that contained specific mutations (Fig. 1B). Replacement of N305A306 with RR in the peptide spanning residues 491–510 had no detectable effect on the binding of mAb 341C, suggesting that neither N305 nor A306 was involved in the epitope for mAb 341C. However, a single mutation of P507 to R resulted in significant loss of binding by mAb 341C (p < 0.05) (Fig. 1C). Similarly, a reduction of binding was observed following the replacement of T509V510 with RR (p < 0.05) (Fig. 1C). The results indicated that residue P507, as well as T509 and/or V510 within peptide 491–510, is important for mAb 341C
Epitope mapping by random peptide phage display. (A) Key residues and the core sequence consensus for antibody binding. Epitope mapping was conducted by screening two phage display libraries, C7C and 12-mer, with mAbs 240C and 540C, respectively. (B) Mapping continuous and discontinuous amino acids involved in antibody binding. Residues identified for each antibody are marked with empty squares, closed or open circles.

Antibody-mediated synergy and interference in SARS-CoV neutralization

The clustering of these epitopes prompted us to examine the functional interaction between their corresponding mAbs measured by virus neutralization assay. Table 1 shows the results obtained from at least three independent neutralization experiments using all the three mAbs independently and/or in combinations. As expected, while mAb 240C did not show any neutralization, mAb 341C and 540C alone neutralized virus infection at titers 2560 and 640, respectively. The neutralization titer rose 4-fold to 10240 when both mAbs 341C and 540C were mixed directly with the virus.

We then asked whether the non-neutralizing mAb 240C could interfere with the neutralizing activities of mAb 341C alone or in combination with mAb 540C (Table 1). As demonstrated by the results from at least three independent neutralization assays, when
both mAbs 240C and 341C were mixed with SARS-CoV, the neutralization titer of mAb 341C was reduced from 2560 to 1280 (Table 1, Direct Mix). Similarly, the presence of 240C also reduced the neutralization titer of mAb 540C from 640 to 320 (Table 1, Direct Mix). The neutralizing activity of the mixture of all three antibodies was lower than that of the two neutralizing antibodies (Table 1, Direct Mix). These results demonstrate that the non-neutralizing mAb 240C can interfere with virus neutralization mediated by mAb 341C and/or mAb 540C.

Finally, we asked whether stepwise mixing of the three mAbs, rather than concomitantly mixing them with the viral stock, would make a difference in neutralizing titers. As shown in Table 1 (Stepwise Mix), there was no significant difference in neutralizing titers when mAbs were added sequentially as compared to adding individual antibodies to the viral inoculum simultaneously. These studies imply that these mAbs have similar binding affinities toward their specific epitopes, and may act in an equilibrium-related manner.

**Discussion**

In this study, we have identified a cluster of epitopes on the spike protein of SARS-CoV. Within this cluster an interplay occurs among non-neutralizing and neutralizing antibodies. First, the antibody-specific epitopes are spatially close to each other. This arrangement creates a unique platform for competition. One of possible mechanisms is that the steric block by non-neutralizing antibodies reduces the amount of virion-bound neutralizing antibody on the spike protein to a suboptimal level of occupancy that can disable neutralization, as concluded by Klasse and co-workers [25,26]. Such condition has been reported for West Nile Virus that antibody-mediated neutralization can be achieved only at relatively high concentrations of virion-bound antibody, whereas an enhancement of infection is observed at lower concentrations through conformational triggering of entry functions or through complement-or Fc receptor-dependent mechanisms [27].
Second, although the epitopes of the neutralizing mAbs 341C and 540C are situated spatially close, partly sharing a single loop structure, they represent two separate interfaces for antibody binding. The synergistic neutralization we observed in our mixing experiments may thus be understood as that these two antibodies are able to bind to their respective epitopes without competition. Finally, by creating closely packed epitope clusters, the virus can elicit non-neutralizing antibodies that will block the binding outside the virus receptor binding site. This finding is not unprecedented; antibodies such as those specific for influenza hemagglutinin neutralize by binding to receptor binding sites or if the binding of each of these mAbs can cause subtle indirect conformational changes in the RBD should provide insights into the mechanism of synergy.

On the other hand, it may also be possible for a host to mount a study on whether mAb 341C or mAb 540C sufficiently blocks other receptor binding sites or if the binding of each of these mAbs can cause subtle indirect conformational changes in the RBD should provide insights into the mechanism of synergy.

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