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Commentary

The role of multiple SARS-CoV-2 viral antigens in a vaccine-induced integrated immune response

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The global pandemic caused by the new coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which emerged at the end of 2019, has led to a public health crisis in which more than 30 million people have been infected and 1 million have died [1,2]. Accelerated vaccine and drug development in different countries and regions (accessed at *ClinicalTrials.gov* as of Oct. 10, 2020) resulted in the rapid entry of several different types of vaccines into clinical trials [3]. Shortly thereafter, a series of clinical data were obtained, especially immunogenic data [4–6]. These studies provide a basis for the development of effective vaccines, but at the same time, they have raised a series of questions regarding the mechanism underlying the immunological response to infection with SARS-CoV-2, the differences in immunogenicity among various types of vaccines and the different immune responses they induce [7]. Our understanding of SARS-CoV-2 is based solely on the limited studies on other members of the coronavirus family [7]. Therefore, the characteristics of the immune response to infection with SARS-CoV-2 should serve as the theoretical basis for the identification of a suitable vaccine antigen. SARS-CoV-2 contains four major structural proteins, namely, spike (S), membrane (M), envelope (E) and nucleocapsid (N). Among these four proteins, S is being used as the leading target antigen in vaccine development because it is responsible for recognition of the host cellular receptor to initiate virus entry. In contrast, N protein packages the viral RNA to form a helical capsid and is essential for virus viability. Although N proteins are highly immunogenic and are expressed abundantly during infection of many coronaviruses family members [8], their complicated role in inducing an immune response is unable to judge its significance as a major target antigen for vaccine. Studies of SARS-CoV and avian coronavirus infectious bronchitis virus showed that N antibodies are not neutralizing nor protecting [9–11]; however, N antibodies or N-specific T cell epitopes have also been reported to protect animals from infection by mouse hepatitis virus, IBV, SARS-CoV and MERS-CoV [12–15]. There are also reports showing that although the crystal structure of the SARS-CoV-2 N protein is similar to that of the SARS-CoV N protein, their surface electrostatic potential characteristics are distinct [16]. These findings might suggest a complicated role of N proteins in different coronavirus infections.

With a microneutralization assay using SARS-CoV-2 live virus and an ELISA kit coated with recombinant S protein, N protein and native purified viral antigen, we were able to detect not only varied titers of neutralizing antibodies (1:16–1:256) but also high titers of anti-S, anti-N and anti-whole-virion antibodies (Fig. 1ab). The presence of these antibodies in the convalescent sera of COVID-19 patients suggests the stimulation of the immune system by various antigen components of SARS-CoV-2 throughout the disease course. This raises the question of whether the antibodies against other viral antigens, especially the N protein as the major nucleocapsid protein, function in the antiviral immune response. The antiviral effect of the anti-N antibody was further observed in a rhesus monkey immunization challenge test using a specially prepared inactivated vaccine containing exposed structural protein components of SARS-CoV-2. In rhesus macaques immunized with two doses of 200, 100 and 20 EUs (ELISA units, the viral antigen concentration determined by ELISA) inactivated vaccine with a 14-day interval, the neutralizing antibody reaction showed a dose-dependent effect of the antigens, and most monkeys were positive on ELISA for the anti-S, anti-N and anti-whole-virion antibodies (Fig. 1cd). The geometric mean titer (GMT) of the neutralizing antibody was 120 in the 200-EU antigen group (N = 4), 32 in the 100-EU antigen group (N = 3) and 2 in the 20-EU antigen group (N = 3). Although the GMT of the neutralizing antibody in two animals was less than 1:4, most animals in the three dose groups had high titers of anti-S, anti-N and anti-whole-virion antibodies from ELISA detection. Further challenge test suggested that all animals showed immune protection upon viral challenge via the nasal route 2 weeks after immunization, in which, viral shedding from the nasal cavity, pharynx and fecal content of immunized animals were found decreasing below the detection limit from 10^4 copies/100ul after 48 h, while those in the positive control were maintained at $10^{3\text{-}4}$ copies/100ul during 7–15 days (Fig. 2a). The detection of viral loads in tissues and organs, especially the respiratory system of animals sacrificed at different time points, also clearly confirmed these findings (Fig. 2b). These results suggest that, at least in protective immunity studies of inactivated vaccines in

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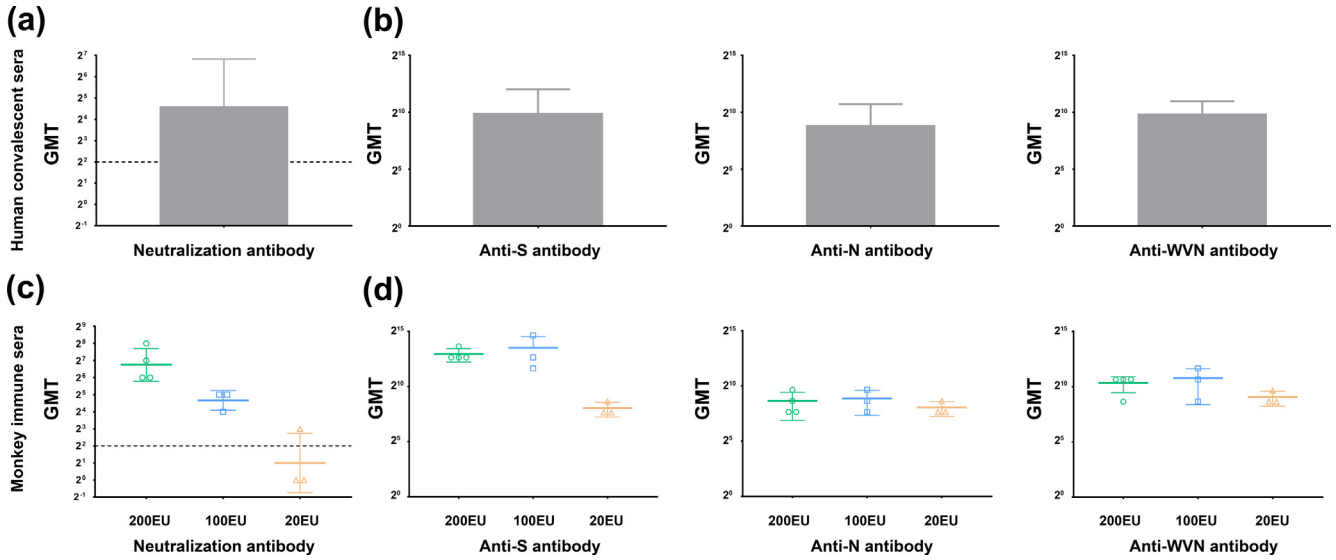

Fig. 1. The antibodies present in the convalescent sera from COVID-19 patients and immunized rhesus macaques by SARS-CoV-2 inactivated vaccine. (a-b) Neutralizing antibodies (a) and anti-S, anti-N and anti-WVN antibodies (b) present in the convalescent sera from COVID-19 patients (N = 71). (c-d) Neutralizing antibodies (c) and anti-S, anti-N and anti-WVN antibodies (d) induced by SARS-CoV-2 inactivated vaccine in rhesus macaques (N = 10). The vaccine contained 200 (N = 4), 100 (N = 3) and 20 (N = 3) enzyme-linked immunosorbent assay (ELISA) units (EUs; the viral antigen concentration determined by ELISA) of inactivated viral antigen adsorbed to 0.25 mg of $Al(OH)_3$ adjuvant and suspended in 0.5 ml of buffered saline in each dose. Blood samples from macaques were obtained on day 7 post boost inoculation. The neutralizing antibody assay was performed via microneutralization assay and the geometric mean titers (GMTs) of antibodies were measured. Neutralizing antibody titers of ≥ 4 were considered positive (dot line). The antibodies against the S protein (spike protein), N protein (nucleocapsid protein) and whole virion (WNV) were quantified via ELISA, and the GMTs were calculated. Diluted serum samples with an optical density (OD) value greater than or equal to 2.1-fold the OD value of the negative control were defined as positive.

rhesus monkeys, the protective efficacy of vaccines does not seem to depend solely on the titer of the neutralizing antibodies but instead might be related to the presence of various types of antibodies in the immunized sera, including anti-N and anti-S antibodies. Based on these findings, we proceeded with a random, double-blinded and control phase 1 clinical trial [17], in which high titers of anti-S, anti-N and anti-whole-virion antibodies associated with the neutralizing antibody raising in low-, medium- and high-dose groups immunized with 0/14-day procedure and the 0/28-day procedure were observed. Combined with the analysis of neutralizing antibody components in the convalescent sera of COVID-19 patients, these data seem to suggest a theory regarding the viral antigen stimulation of the immune system over the course of an infection with SARS-CoV-2: As we know, the virus infects the human body through the respiratory tract, which means that the binding of the viral S protein RBD to the surface receptor ACE2 on respiratory tract epithelial cells is the first step in initiating the viral infection [18], and, this binding mediates viral entry into the host cell followed by the separation of the S protein into the S1 and S2 subunits [19]. The S2 subunit mediates the fusion between the viral envelope and the host cell membrane, allowing the virus nucleocapsid to enter the cell without the envelope [20]. This fact suggests that the nucleocapsid antigen component of the virus, which apparently includes the N antigen, is the first component to be recognized by the pattern recognition receptor (PRR) in the respiratory tract epithelium as the innate immune response to the pathogen-associated molecular pattern (PAMP). The NF-kB pathway activated by this antigen signaling transcribes and expresses immune signal molecules specifically stimulating different innate immune cells in the epithelium, which in turn leads to the activation of different innate immune cells, especially dendritic cells, and the phagocytosis of infected cells as well as the transfer of antigen-stimulating signals to the T cells via antigen presentation. This whole process might suggest that the signals of the N antigen, S antigen and other viral antigens may play important roles in the activation of the adaptive immune system and the formation of antiviral immunity. Our previous study suggested that during the process of infecting 16HBE human bronchial epithelial cells with SARS-CoV-2, significant changes occur in the transcription levels of different immune signaling molecules during the virus proliferation and release from host cells, of which IL-22 is the most clearly affected [21]. In addition, SARS-CoV-2 has been shown to infect dendritic cells as it does epithelial cells in our related preliminary studies (in submission). Based on these data, we can reasonably conclude that the design of the antigen in a vaccine against SARS-CoV-2 should consider the presence of other viral antigens, especially the N antigen, in addition to the S antigen as the main component inducing neutralizing antibodies.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [The sponsor of the study played no role in the study design, data and sample collection, data processing, or report writing. The corresponding author had full access to all the data generated by the study and takes full responsibility for the final submission for publication].

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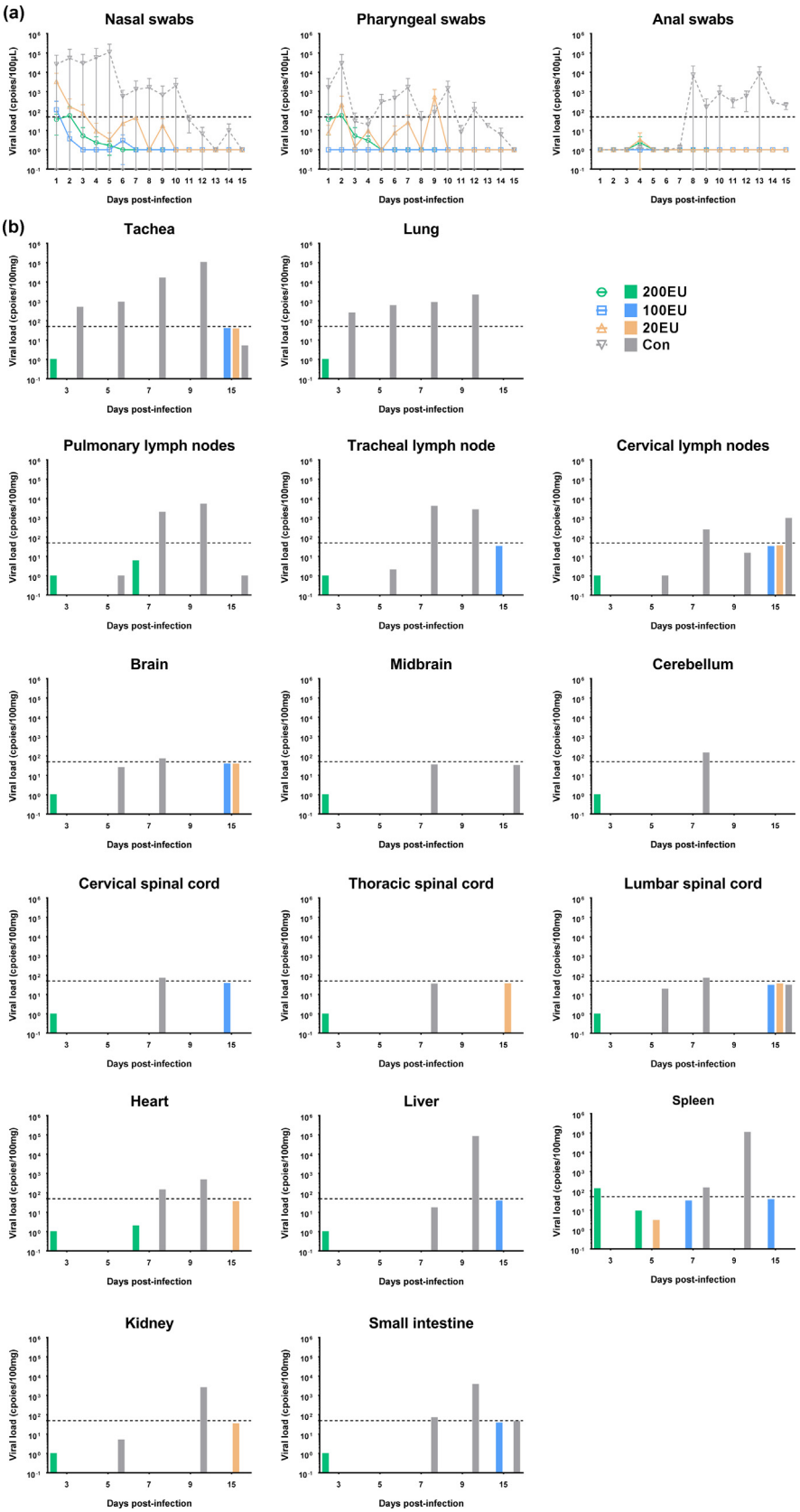

Fig. 2. The integrated immune response elicited by the inactivated vaccine can limit viral replication during viral challenge in rhesus macaques. Viral loads in nasal swabs, pharyngeal swabs and anal swabs from monkeys immunized with the inactivated vaccine (200 EUs, 100 EUs and 20 EUs; N = 10) after infection with SARS-CoV-2. Copies of less than 50 (dotted lines) were considered negative. The results are shown as the mean ± SD. (a) Viral loads in respiratory system (trachea, lung), lymph nodes (pulmonary lymph nodes, tracheal lymph nodes and cervical lymph nodes), nervous system (brain, midbrain, cerebellum, cervical spinal cord, thoracic spinal cord, lumbar spinal cord) and other major organs (heart, liver, spleen, kidney and small intestine) of macaques immunized with the inactivated vaccine (200 EUs, 100 EUs and 20 EUs; N = 10) after infection with live virus. Copies of less than 50 (dotted lines) were considered negative.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2021.03.067.

References

[1] Gupta P, Goyal K, Kanta P, Ghosh A, Singh MP. Novel 2019-coronavirus on new year's Eve. Indian J Med Microbiol. 2019;37(4):459–77.

[2] Johns_Hopkins_University. COVID-19 Dashboard by the Center for Systems Science and Engineering (CSSE) at Johns Hopkins University (JHU). 2020. (Accessed Oct 10, 2020, at https://www.arcgis.com/apps/opsdashboard/index.html#/bda7594740fd40299423467b48e9ecf6.)

[3] Rego GNA, Nucci MP, Alves AH, Oliveira FA, Marti LC, Nucci LP, et al. Current Clinical Trials Protocols and the Global Effort for Immunization against SARS-CoV-2. Vaccines (Basel) 2020;8(3):474.

[4] Folegatti PM, Ewer KJ, Aley PK, Angus B, Becker S, Belij-Rammerstorfer S, et al. Safety and immunogenicity of the ChAdOx1 nCoV-19 vaccine against SARS-CoV-2: a preliminary report of a phase 1/2, single-blind, randomised controlled trial. Lancet 2020;396:467–78.

[5] Xia S, Duan K, Zhang Y, Zhao D, Zhang H, Xie Z, et al. Effect of an Inactivated Vaccine Against SARS-CoV-2 on Safety and Immunogenicity Outcomes: Interim Analysis of 2 Randomized Clinical Trials. JAMA 2020;324(10):951–60.

[6] Zhu FC, Guan XH, Li YH, Huang JY, Jiang T, Hou LH, et al. Immunogenicity and safety of a recombinant adenovirus type-5-vectored COVID-19 vaccine in healthy adults aged 18 years or older: a randomised, double-blind, placebo-controlled, phase 2 trial. Lancet 2020;396(10249):479–88.

[7] Subbarao K. SARS-CoV-2: A New Song Recalls an Old Melody. Cell Host Microbe. 2020;27(5):692–4.

[8] Cong Y, Ulasli M, Schepers H, Mauthe M, V'Kovski P, Kriegenburg F, et al. Nucleocapsid Protein Recruitment to Replication-Transcription Complexes Plays a Crucial Role in Coronaviral Life Cycle. J Virol. 2020;94(4):e01925–19.

[9] Yasui F, Kai C, Kitabatake M, Inoue S, Yoneda M, Yokochi S, et al. Prior immunization with severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV) nucleocapsid protein causes severe pneumonia in mice infected with SARS-CoV. J Immunol. 2008;181(9):6337–48.

[10] Deming D, Sheahan T, Heise M, Yount B, Davis N, Sims A, et al. Vaccine efficacy in senescent mice challenged with recombinant SARS-CoV bearing epidemic and zoonotic spike variants. PLoS Med. 2006;3(12):e525. e525.

[11] Ignjatovic J, Sapats S. Identification of previously unknown antigenic epitopes on the S and N proteins of avian infectious bronchitis virus. Arch Virol. 2005;150(9):1813–31.

[12] Zhao J, Zhao J, Mangalam AK, Channappanavar R, Fett C, Meyerholz DK, et al. Airway Memory CD4(+) T Cells Mediate Protective Immunity against Emerging Respiratory Coronaviruses. Immunity 2016;44(6):1379–91.

[13] Nakanaga K, Yamanouchi K, Fujiwara K. Protective effect of monoclonal antibodies on lethal mouse hepatitis virus infection in mice. J Virol. 1986;59(1):168–71.

[14] Lecomte J, Cainelli-Gebara V, Mercier G, Mansour S, Talbot PJ, Lussier G, et al. Protection from mouse hepatitis virus type 3-induced acute disease by an anti-nucleoprotein monoclonal antibody. Brief report. Arch Virol. 1987;97(1–2):123–30.

[15] Collisson EW, Pei J, Dzielawa J, Seo SH. Cytotoxic T lymphocytes are critical in the control of infectious bronchitis virus in poultry. Dev Comp Immunol 2000;24(2–3):187–200.

[16] Kang S, Yang M, Hong Z, Zhang L, Huang Z, Chen X, et al. Crystal structure of SARS-CoV-2 nucleocapsid protein RNA binding domain reveals potential unique drug targeting sites. Acta Pharm Sin B. 2020;10(7):1228–38.

[17] Pu J, Yu Q, Yin Z, Zhang Y, Li X, Li D, et al. An in-depth investigation of the safety and immunogenicity of an inactivated SARS-CoV-2 vaccine. medRxiv. 2020;2020(09). pp. 27.20189548.

[18] Wang Q, Zhang Y, Wu L, Niu S, Song C, Zhang Z, et al. Structural and Functional Basis of SARS-CoV-2 Entry by Using Human ACE2. Cell 2020;181(4):894–904.e9.

[19] Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, et al. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature 2003;426(6965):450–4.

[20] Supekar VM, Bruckmann C, Ingallinella P, Bianchi E, Pessi A, Carfí A. Structure of a proteolytically resistant core from the severe acute respiratory syndrome coronavirus S2 fusion protein. Proc Natl Acad Sci U S A. 2004;101(52):17958–63.

[21] Liao Y, Li X, Mou T, Zhou X, Li D, Wang L, et al. Distinct infection process of SARS-CoV-2 in human bronchial epithelial cells line. J Med Virol. 2020;92(11):2830–8.