Intramuscular injection of a mixture of COVID-19 peptide vaccine and tetanus vaccine in horse induced neutralizing antibodies against authentic virus of SARS-CoV-2 Delta variant

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Abstract

Peptide vaccine is not effective due to its low immunogenicity. To improve the efficacy of peptide vaccine against COVID-19, a novel method was developed by mixing a COVID-19 peptide vaccine with a tetanus vaccine. In this study, intramuscular injection of a mixture of COVID-19 peptide vaccine and tetanus vaccine twice, i.e., first dose on day 0 and second dose on day 21, induced neutralizing antibodies against authentic virus of SARS-CoV-2 Delta variant in a horse. Horse serum of day 35, i.e., two weeks after the second dose, neutralized authentic virus of SARS-CoV-2 Delta variant, equal to half effectiveness of human serum from vaccinees of Moderna COVID-19 vaccine. However, neither horse serum nor human serum neutralized Omicron variant authentic virus. No side effects were observed after each dose. This study indicates intramuscular injection of a mixture of COVID-19 peptide vaccine and tetanus vaccine may work in humans to improve peptide vaccine efficacy against SARS-CoV-2.

Introduction

Peptide-based synthetic vaccines, also called epitope vaccines, are subunit vaccines made from peptides. The peptides mimic B-cell and T-cell epitopes of the antigen to trigger B-cell and T-cell immune responses against the antigen of infectious or non-infectious pathogens. Epitope identification based on amino acid sequence of the antigen, adjuvant selection, and vaccination method are pivotal for a peptide vaccine to induce strong immune responses [1]. Peptide vaccine is safe, easy to produce, and highly specific. The disadvantage of peptide vaccine is low efficacy, resulting from its low immunogenicity. Although many clinical trials have been conducted, the progress is slow [2,3]. United States Food and Drug Administration (FDA) has not yet approved or authorized any peptide vaccine for human use against any disease. Therefore, a method for enhancing the immune response induced by peptide vaccine is needed in order to improve vaccine efficacy.

In this proof-of-concept study, a novel method to enhance the immune response induced by Coronavirus disease 2019 (COVID-19) peptide vaccine, i.e., mixing COVID-19 peptide vaccine with tetanus vaccine, was developed in order to improve efficacy of COVID-19 peptide vaccine in horse. Our objective is to vaccinate a person against COVID-19 with the mixture of a COVID-19 peptide vaccine and a tetanus toxoid-containing vaccine in order to improve efficacy of COVID-19 peptide vaccine. Our hypothesis of mixing COVID-19 peptide vaccine with tetanus vaccine is to take advantage of both the adjuvant and the tetanus toxoid in a tetanus vaccine, i.e., using the adjuvant of a tetanus vaccine as the adjuvant for a COVID-19 peptide vaccine and harnessing the cytokines secreted by anti-tetanus toxoid T helper cells to enhance B cell and cytotoxic T lymphocyte responses to a COVID-19 peptide vaccine. There is no similar study that has been conducted before [4–6].

The data of safety and efficacy of vaccination through intramuscular injection of a mixture of COVID-19 peptide vaccine and tetanus vaccine in a horse presented here indicate this novel method may be used in humans to improve vaccine efficacy against COVID-19. If successful in human too, this strategy may further be applied to peptide vaccines against other human diseases such as cancer and AIDS.

Materials and methods

COVID-19 peptide vaccine

Forty-five peptides that are components of the spike (S) protein of severe acute respiratory syndrome-related coronavirus 2
mutations are the following:

due positions in the 1273-amino-acid S protein, and the related
Amino acid sequences of these 45 peptides, their amino acid resi-

commercial laboratory (GenScript, Inc., Piscataway, NJ, USA).

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19mer.

peptide#39. SYQTQNSPRRASVAS (673–689), 15mer.

peptide#42. QTQTNSHRARSVAS (675–689, P681H), 15mer.

peptide#43. LPDPSKPSARSIEDLF (806–823), 18mer.

peptide#44. YEYQIKPWVYIV (1206–1217), 12mer.

peptide#45. TSCSCLKGCSCGCS (1238–1252), 15mer.

Each peptide was chemically synthesized at a purity of 85% and
a quantity of 4 mg. These 45 peptides were then mixed to make a
180 mg multi-peptide cocktail, equally divided into two parts, and
stored in two vials. Each vial contains 90 mg multi-peptide cocktail,
tail, i.e., the COVID-19 peptide vaccine used in this study. These
two vials were used as two doses given 3 weeks apart to a horse.
Three of these 45 peptides match one mutation of Delta variant.
Eleven of these 45 peptides match five mutations of Omicron vari-
ant. The length range of these 45 peptides is from 12mer to 33mer
and these peptides correspond to B-cell or T-cell epitopes of S pro-
tein of SARS-CoV-2 [7–12].

Tetanus vaccine

A commercially available tetanus toxoid vaccine ( Prestige Teta-
nus, Merck Animal Health/Intervet Inc., Rahway, NJ, USA) was used
in this study. This equine tetanus vaccine contains tetanus toxoid
and Havlogen adjuvant.

Mixture of COVID-19 peptide vaccine and tetanus vaccine

Approximately 90 mg of COVID-19 peptide vaccine, i.e., 2 mg
for each of the 45 peptides, was mixed with 3 ml phosphate buf-
fered saline (PBS) and 1 ml tetanus vaccine to make a homogenous
suspension for horse vaccination. The amount of tetanus vaccine
was 1 regular dose for a horse as recommended by the manufac-
turer. The volume of PBS was the minimum amount of PBS
required to fully suspend the peptide powder, which was then
mixed with the tetanus vaccine.

Horse vaccination and horse serum collection

An adult healthy horse was vaccinated with a mixture of
COVID-19 peptide vaccine and tetanus vaccine, i.e., 90 mg peptide,
3 ml PBS, and 1 ml tetanus vaccine, through intramuscular injec-
tion in the neck muscle twice, i.e., first dose on day 0 and second
dose on day 21. Blood was drawn from the jugular vein on days
0 and 21 (pre-vaccination), 2 and 23 (48 h post- vaccination) for
measurement of complete blood count (CBC) and acute phase pro-
teins fibrinogen and serum amyloid A. Blood was also collected on
day 35, serum was isolated and stored at −80 °C. Horse serum of
day 35, which was collected two weeks after the second dose,
should have the highest titer of neutralizing antibodies against SARS-CoV-2 original strain and its variants. The protocol of this
horse experiment was approved by Institutional Animal Care and
Use Committee (IACUC) of the University of Pennsylvania. This
horse experiment was conducted at the Department of Clinical
Studies-New Bolton Center, University of Pennsylvania School of
Veterinary Medicine.

Neutralization assay using SARS-CoV-2 authentic virus for the
measurement of neutralizing antibody titer against SARS-CoV-2
original strain and its variant in horse serum

(1). SARS-CoV-2 strains

NR-55612 virus (Lineage B.1.617.2, Delta Variant) and NR-
56462 virus (Lineage B.1.1.529, Omicron Variant) were obtained
through the Biodefense and Emerging Infectious Researches
Resources Repository (BEI Resources), NIAID, NIH, USA.

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peptide#1. LFLPFFSVTWHAIGSTNGKRDNPVLPFND (54–68 + 71–88, 69/70 deletion), 33mer.

peptide#2. NVTWHAIGSTNGKRDNPVLPFND (54–68 + 71–80, 69/70 deletion), 18mer.

peptide#3. KVCEFQFCNDPFLGYYKHKNNSWEMESFRRYVS (129–161), 33mer.

peptide#4. QFCNDPFLGYYKHKNNSWEMESFRRYVS (134–141 + 155–161), 15mer.

peptide#5. GDSSGWTAGAAAYVGYLQPRFLKLKYENGT (252–284), 33mer.

peptide#6. GEFVNAVTSFYAWNRKRSNCVADYSVLYN (339–371), 33mer.

peptide#7. DLCTFNVYADSFVIRGDEVR (389–408), 20mer.

peptide#8. CFVIRGDEVRQPAC (C + 400–413 + D), 16mer.

peptide#9. VIRGDEVRQIAQPTGNNYKLPDDFTGCCV (401–433, K417N), 33mer.

peptide#10. VIRGDEVRQIAQPTGNNYKLPDDFTGCCV (401–433, K417T), 33mer.

peptide#11. RGDEVRQIAQPTGNNYKLPDDFTGCCV (401–433), 14mer.

peptide#12. YQAGSTPCNGVEGFNCYFPLQS (462–500, E484K), 33mer.

peptide#13. VGGNYLYRLFRKSNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#14. VGGNYLYRLFRKSNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#15. VGGNYLYRLFRKSNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#16. SNLDSKVGNYLYRLFR (438–456), 19mer.

peptide#17. SNLDSKVGNYLYRLFR (438–456), 19mer.

peptide#18. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#19. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#20. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#21. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#22. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#23. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#24. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#25. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#26. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#27. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#28. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#29. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#30. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#31. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#32. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#33. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#34. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#35. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#36. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#37. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#38. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#39. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#40. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#41. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#42. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#43. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#44. SNLKPFERDISTEIYQAGSC (445–477), 33mer.

peptide#45. SNLKPFERDISTEIYQAGSC (445–477), 33mer.
through World Reference Center for Emerging Viruses and Arboviruses, The University of Texas Medical Branch.

(2). Horse serum

Horse serum of day 35, i.e., two weeks after the second dose of a mixture of COVID-19 peptide vaccine and tetanus vaccine, was used for the study.

(3). Human serum

NRH-17846 (Pooled human serum sample from vaccinees of Moderna COVID-19 vaccine) was obtained through BEI Resources, NIAID, NIH.

(4). Rabbit polyclonal anti-SARS-CoV-2 spike glycoprotein antibody

NR-52947 [Polyclonal anti-SARS-related coronavirus 2 spike glycoprotein (IgG, Rabbit)] was obtained through BEI Resources, NIAID, NIH. A recombinant form of the spike glycoprotein (residues 16 to 1213 with mutations R683A and R685A; histidine-tagged) from SARS-CoV-2 was used to immunize rabbits to produce this polyclonal antibody.

(5). Procedure

To measure the titer of neutralizing antibodies against SARS-CoV-2 original strain and its variants in horse serum of day 35, human serum (NRH-17846) and rabbit antibody (NR-52947) were used as controls. Briefly, SARS-CoV-2 original strain (USA-WA1/2020), Delta variant (NR-55612), or Omicron variant (NR-56462) stocks were prepared by passing the virus in Vero E6 cells using test media of MEM supplemented with 2 % FBS and 50 μg/mL gentamicin. Horse serum of day 35, human serum, or rabbit antibody was serially diluted using eight 2-fold dilutions in test media so that the starting (high) concentration was a 1/2 dilution. Virus was prepared and added in an equal volume to each prepared dilution so that the final MOI was approximately 0.01. Eight tubes containing virus with no test sample and eight tubes containing only test media were included as virus controls and cell controls, respectively. Sample and virus mixtures were incubated at room temperature for 1 h. Following the incubation, each dilution plus virus mixture was added to 2 wells of a 96-well plate with 80–100% confluent Vero E6 cells. Plates were incubated at 37 ± 2 °C, 5 % CO₂. On day 6 post-infection, once virus cytopathic effect (CPE) was >80 % in virus control wells, plates were visually inspected for CPE in all wells. The neutralizing antibody titer was determined as the reciprocal of the highest dilution where no viral CPE was present in either of the 2 duplicate wells (complete neutralization). This neutralization essay was conducted in Institute for Antiviral Research, Utah State University.

Results

Vaccination with a mixture of COVID-19 peptide vaccine and tetanus vaccine through intramuscular injection is safe

After each of the two doses, the horse was monitored for immediate anaphylactic reaction. The horse was further monitored over the next 48 h for fever or swelling at the injection site. There were no adverse effects observed. The complete blood count (CBC) performed pre-vaccination was normal, with mild changes seen 48 h after each vaccination (Table 1).

Most of the parameters in Table 1 remained in normal range and there were no significant changes after vaccination other than normal biological variation. However, we noted that after both vaccine doses, the fibrinogen did rise, just barely out of normal range. Fibrinogen is an acute phase protein that increases with an immune response to inflammation. This is a mild rise but suggests at least that the immune system recognized and responded to the antigens in the vaccine. Serum amyloid A is another acute phase protein that increases with inflammation. While it did not rise significantly after the first vaccine, it did go up after the booster, again indicating that the booster dose stimulated an immune response. Also, the neutrophil count (a white blood cell associated with inflammation) also rose after both the initial dose and the booster, although it did not rise above normal after the initial. In summary, these results indicate a mild inflammatory response that was most noticeable after the booster, but nothing out of the ordinary and it is a response that might be expected following vaccination.

Vaccination with a mixture of COVID-19 peptide vaccine and tetanus vaccine is effective against authentic virus of SARS-CoV-2 Delta variant

Horse serum of day 35, i.e., two weeks after the second dose, pooled human serum from vaccinees of Moderna COVID-19 vaccine (NRH-17846), and rabbit polyclonal anti-SARS-CoV-2 spike glycoprotein IgG antibody (NR-52947) were measured for the neutralizing antibody titer against authentic viruses of Delta variant (NR-55612), Omicron variant (NR-56462), and SARS-CoV-2 original strain (USA-WA1/2020). Results of neutralization assay are shown in Table 2.

Table 2 shows neutralizing antibody titer against Delta variant authentic virus is 8 in horse serum and 16 in human serum. In other words, horse serum of day 35 neutralized SARS-CoV-2 Delta variant authentic virus, equal to half effectiveness of human serum from vaccinees of Moderna COVID-19 vaccine. Table 2 further...
shows neither horse serum nor human serum neutralized Omicron variant authentic virus. This result is similar to the finding that Omicron variant escapes the majority of existing SARS-CoV-2 neutralizing antibodies by other researchers [13–15]. Cost of testing precluded testing pre-vaccination sera, but presumably the horse, which is not a natural host to SARS-CoV-2, was seronegative at the onset.

In conclusion, intramuscular injection of a mixture of COVID-19 peptide vaccine and tetanus vaccine appears both safe and effective at inducing a neutralizing antibody response.

### Discussion

The purpose of mixing COVID-19 peptide vaccine with tetanus vaccine is to take advantage of both the adjuvant and the tetanus toxoid in a tetanus vaccine in order to enhance the immune response induced by COVID-19 peptide vaccine. American Association of Equine Practitioners (AAEP) recommends all adult horses are initially vaccinated for tetanus through intramuscular injection twice, three to six weeks apart, using tetanus toxoid and then given a booster annually. Centers for Disease Control and Prevention (CDC) recommends tetanus vaccination for all babies and children, preteens and teens, and adults. Tetanus vaccination is currently often administered as combination vaccines through intramuscular injection. Four kinds of tetanus toxoid-containing vaccines used today protect against tetanus, all of which also protect against other diseases: Diphtheria, tetanus, and pertussis (DTaP), Diphtheria and tetanus (DT), Tetanus, diphtheria, and pertussis (Tdap), Tetanus and diphtheria (Td). Babies and children younger than 7 years old receive DTaP or DT, while older children and adults receive Tdap and Td. CDC recommends all adults to get a shot of Tdap or Td every 10 years. Therefore, one of these four tetanus toxoid-containing vaccines, i.e., DTaP, DT, Tdap, or Td, can be used in people at any age.

Prestige tetanus vaccine used in this study is effective for the vaccination of healthy horses, cattle, swine and sheep 6 months of age or older against tetanus. It contains tetanus toxoid and Havlogen adjuvant. Havlogen is an emulsive, lipid-based, carbopolar polymer cross-linking adjuvant. Havlogen stimulates the immune system to produce high, long-lasting levels of protection through the slow release and gradual absorption of antigen. It is unclear whether Havlogen adjuvant played a key role in the present study and whether it can be replaced by another adjuvant.

The SARS-CoV-2 S protein is 1273 amino acids long and it consists of a signal peptide (amino acid residues 1–13) located at the N-terminus, the S1 subunit (14–685 residues), and the S2 subunit (686–1273 residues); the last two regions are responsible for receptor binding and membrane fusion respectively [7,16–18]. Novavax vaccine “NVX-CoV2373” is the first approved recombinant protein-based vaccine to SARS-CoV-2 [6]. NVX-CoV2373 consists of full-length, stabilized, prefusion, recombinant S protein components combined with a saponin-based adjuvant, Matrix-M. It elicits both B-lymphocyte and T-lymphocyte immune responses to the SARS-CoV-2 S protein, including viral neutralizing antibodies. Also, the full-length S protein in this vaccine has common epitopes that could protect against all the SARS-CoV-2 viral variants. Our 45 peptides correspond to many parts of the SARS-CoV-2 S protein, i.e., from residue 54 to residue 1252, and the length of these 45 peptides varies between 12mer and 33mer. Thus, our COVID-19 peptide vaccine should elicit both B-cell and T-cell immune responses to the SARS-CoV-2 S protein, including viral neutralizing antibodies [2,5,6]. As shown below, 17 of the 45 peptides match 8 mutations in the SARS-CoV-2 S protein. Thus, our COVID-19 peptide vaccine should protect against some variants of SARS-CoV-2.

The 45 peptides used in this study were originally synthesized to match S protein of SARS-CoV-2 original strain and its Alpha, Beta, and Gamma variants, and the horse vaccination was finished before the appearance of Delta and Omicron variants. Twenty-eight of the 45 peptides match the original strain. Seventeen of the 45 peptides, i.e., peptides #1, #2, #9, #10, #12, #20, #22, #27, #30, #32, #33, #35, #36, #38, #39, and #42, match 8 mutations of Alpha, Beta, and Gamma variants, i.e., 69/70 deletion, K417N, K417T, E484K, N501Y, N507D, D614G, P681H. There are shared mutations among Alpha, Beta, and Gamma variants and the details are the following:

1. Eleven peptides match 5 mutations of Alpha variant, i.e., 69/70 deletion (peptides #1 and #2), N501Y (peptides #27 and #30), A570D (peptides #32 and #33), D614G (peptides #35, #36, and #38), P681H (peptides #39 and #42).
2. Night peptides match 4 mutations of Beta variant, i.e., K417N (peptides #9 and #12), E484K (peptides #20 and #22), N501Y (peptides #27 and #30), D614G (peptides #35, #36, and #38).
3. Eleven peptides match 5 mutations of Gamma variant, i.e., K417N (peptides #9 and #12), K417T (peptides #10 and #13), E484K (peptides #20 and #22), N501Y (peptides #27 and #30), D614G (peptides #35, #36, and #38).

Three of these 45 peptides (peptides #35, #36, and #38) happened to match 1 mutation of Delta variant, i.e., D614G. Eleven of these 45 peptides happened to match 5 mutations of Omicron variant, i.e., 69/70 deletion (peptides #1 and #2), K417N (peptides #9 and #12), N501Y (peptides #27 and #30), D614G (peptides #35, #36, and #38), P681H (peptides #39 and #42). Therefore, we measured neutralizing antibody titers against Delta and Omicron variants in horse serum. We found that horse serum of day 35, i.e., two weeks after the second dose, neutralized authentic virus of SARS-CoV-2 Delta variant, equal to half effectiveness of human serum from vaccinees of Moderna COVID-19 vaccine. However, neither horse serum nor human serum neutralized Omicron variant authentic virus.

Our novel vaccine strategy, i.e., taking advantage of both the adjuvant and the tetanus toxoid in a tetanus vaccine, is different from other COVID-19 peptide vaccine strategies published in the literature, i.e., focusing on B-cell and T-cell epitope identification and adjuvant development [2–5,19–23]. Furthermore, we used authentic viruses in neutralization assay whereas others did not. We believe our COVID-19 peptide vaccine induced T cell response in the horse, analysis of which will be the focus of future research.
Because some of these 45 peptides correspond to T-cell epitopes of the SARS-CoV-2 S protein [19,24].

As 3 peptides in this 45-peptide cocktail, i.e., peptides #35, #36, and #38, match 1 mutation of Delta variant, i.e., D614G, this mutation site may be important for the development of effective COVID-19 vaccine against SARS-CoV-2 original strain and its past, current and future variants [11]. This 3-peptide cocktail, mixed with a tetanus vaccine, can be used to vaccinate horses or mice to produce neutralizing antibodies against the Delta variant. If successful too, a clinical trial should be conducted. As 11 peptides in this 45-peptide cocktail match 5 mutations of Omicron variant and the Omicron variant has 30 mutations, the remaining 25 mutations may be important for the development of effective COVID-19 vaccine against Omicron variant. These experiments are the forthcoming studies.

The results of the present study show intramuscular injection of a mixture of COVID-19 peptide vaccine and tetanus vaccine induced neutralizing antibodies against SARS-CoV-2 Delta variant authentic virus in a horse, demonstrating successful proof of concept. The results also show intramuscular injection of a mixture of COVID-19 peptide vaccine and tetanus vaccine was safe in this horse, offering reassurance on safety of both peptide vaccine and tetanus vaccine. These safety and efficacy data in this horse indicate intramuscular injection of a mixture of COVID-19 peptide vaccine and tetanus toxoid-containing vaccine, i.e., DTA, DT, Tdap, or Td, may be used for clinical trial as a safe and effective COVID-19 vaccine in people of all ages. The data also indicate administration of a mixture of a peptide vaccine against other disease such as cancer, AIDS, hepatitis B, Alzheimer’s disease, allergy, and autoimmune disease and a tetanus toxoid-containing vaccine may enhance the immune responses against other disease. The data further indicate administration of a mixture of another type of COVID-19 vaccine, i.e., mRNA, adenoviral vector, inactivated whole virus, or recombinant protein vaccine, and a tetanus toxoid-containing vaccine may enhance the immune responses against COVID-19 and even reduce vaccine side effects if a smaller dose of these COVID-19 vaccines is used in the mixture. Since 70.3% of the world population has received at least one dose of a COVID-19 vaccine, DTA, DT, Tdap, or Td may be used as a universal booster vaccine to reduce the severity of a COVID-19 breakthrough infection.

The authors acknowledge the limitations of this study, in that only one horse was used in this proof-of-concept pilot.

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Weiwen Deng has a relationship with Behring Therapeutics, Inc. that includes board membership. Weiwen Deng is inventor on a U.S. provisional patent application (63/345,049) related to the methodology described in the present study. The remaining author declares no competing interests.

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