Citrulline as a novel adjuvant candidate for vaccines

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ABSTRACT

For a long time, many types of vaccines have been useful for the prophylaxis of many infectious diseases. Thus far, many adjuvants that enhance the effects of vaccines have been explored. However, very few adjuvants are being used for humans worldwide. In this study, we investigated the adjuvant activity of various substances, and found citrulline to have high potential as an adjuvant. Citrulline is a type of amino acid present in the body of many organisms. A number of biological activities of citrulline have been reported; however, no adjuvant activity has been reported thus far. Aluminum salts, which are commonly used as adjuvants are not water soluble; therefore, some difficulties are encountered while using them as vaccine adjuvants. Citrulline is easy to use because of its water solubility. In this study, we showed for the first time the adjuvant activity of citrulline by using viral antigens and amyloid \(\beta\) peptide. Water-soluble citrulline, which is present in our body, is a potential adjuvant candidate.

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Vaccines have played very important roles in protecting and overcoming infectious diseases in humans to date and their roles are increasingly becoming larger. Adjuvants are substances added to vaccines to enhance the immunogenicity of the vaccines, and have been used in production of human vaccines for over 90 years. They are highly advantageous in reducing doses and the number of administrations of a vaccine. Therefore, a variety of studies have been conducted on adjuvants (6, 20–22, 28, 36). Many substances have been investigated for their adjuvant potential, but only aluminum salts continue to be used widely in the world.

Aluminum salts are safe and inexpensive. However, they are not ideal adjuvants from the viewpoint of convenience because it is hard to mix them uniformly with an antigen due to their insolubility and to use them in a device for nasal or transdermal administration. Other compounds including squalene-based oil-in-water emulsions and monophosphoryl lipid have been licensed as adjuvants (3, 32). Although these compounds may exert a strong adjuvant activity, they are associated with a high incidence of adverse reactions at the injected sites and are insoluble in water, which is a disadvantage (31, 33). Therefore, studies are underway worldwide to identify easy-to-use adjuvants with few side reactions. We have previously investigated the adjuvant activity of convenient substances such as amino acids and sugars, which are considered safe. Then, we found that citrulline has adjuvant activity. Citrulline was discovered in watermelon in Japan in 1914 and the name was derived from \(\textit{Citrullus vulgaris}\), the scientific name for watermelon (13). This amino acid does not constitute proteins existing in an organism, but is one of the intermediates that constitute the urea cycle and is prevalent among animals, in particular, mammals (11). Citrulline can be synthesized from arginine \textit{in vivo}, and nitrogen oxide known to have a vasodilating activity is released in the process. On the other hand, citrulline also regenerates...
arginine by a condensation reaction with aspartic acid in the urea cycle. Furthermore, it exhibits beneficial activities such as acceleration of the ammonium metabolism (19), improvement of blood flow due to vasodilatation (24), decrease in blood pressure (9), neurotransmission (25), and elimination of active oxygen (17). Therefore, citrulline has been commercially available as a health supplement in Japan, the USA, and Europe. Arginine, like citrulline, is one of the intermediates in the urea cycle and is known to have adjuvant activity (26, 30). However, the adjuvant activity of citrulline has not been reported thus far. In this study, we report the adjuvant activity of citrulline in comparison with aluminium salt and arginine, which to our knowledge is the first such report.

MATERIALS AND METHODS

Reagents and antigens. Citrulline solution for use as an adjuvant was prepared as described below. L-Citrulline (Sigma–Aldrich, MO, USA) was dissolved in distilled water (Otsuka Pharmaceutical, Tokyo, Japan) to achieve a final concentration of 80 mg/mL for injection. The solution was aseptically filtered with 0.22-μm membrane filter and stored at −20°C until use.

Hemagglutinin (HA) of influenza virus (Strain A/Solomon Islands/3/2006), inactivated Japanese encephalitis virus (JEV, Beijing-1 strain), and hepatitis B virus surface antigen (HBs) were obtained from KM Biologics Co., Ltd. (Kumamoto, Japan). Modified external domain of the matrix protein 2 (M2e) peptides (PM10) of influenza A virus and amyloid β peptides (Aβ1–28-Cys) were synthesized with their original peptides at BEX (Tokyo, Japan) and Sigma–Aldrich, respectively, and prepared according to previously published reports (15, 18). Aluminum salt, which was used as a control adjuvant, was purchased from Brenntag Biosector (Frederikssund, Denmark).

Animals. Female BALB/c mice, female ddY mice, and male C57BL/6 mice were used in this study. The mice were bred under specific pathogen-free conditions prior to the experiments. One week following the preliminary breeding, mice were subjected to immunization studies. All procedures for animal experiments were approved by our institutional animal care and use committee, and performed according to the animal experimentation regulations.

Immunization. The mice were divided into several groups of three to six mice each according to the study and were immunized with antigens subcutaneously with or without various doses of citrulline. After immunization, blood samples were obtained and antibodies against antigens in the animals’ sera were measured using enzyme-linked immunosorbent assay (ELISA) or corresponding assays.

Assay of antibodies. ELISA for antibodies against HA was performed as mentioned below. HA was coated on the ELISA plate (Thermo Fisher Scientific, MA, USA). After blocking with Blocker™ Casein (Thermo Fisher Scientific) in phosphate-buffered saline (PBS), serially diluted antisera (200- to 25600-fold) with Blocker™ Casein in PBS were added and HA-specific IgG titers were measured with horseradish peroxidase-conjugated anti-mouse IgG (Thermo Fisher Scientific) and substrate BioFX (TMB) (Surmodics, MN, USA). The antigen-specific IgG titers were determined by absorbance at 450 nm.

ELISA for antibodies against inactivated JEV was performed as follows. The Japanese encephalitis vaccine antigen, same as the one used for the immunization, was coated on the ELISA plate (Thermo Fisher Scientific). After blocking with Block Ace (DS Pharma Biomedical, Osaka, Japan), serially diluted antisera (1600- to 204800-fold) with Block Ace were added and JEV-specific IgG titers were measured with horseradish peroxidase-conjugated anti-mouse IgG (American Qualax, CA, USA) and substrate TMB+ (Dako, Glostrup, Denmark). The JEV-specific IgG titers were determined by absorbance at 450 nm.

Antibodies against HBs were measured with the E test “TOSOH” II (HBsAb) (TOSOH, Tokyo, Japan) according to the manufacturer’s instruction. ELISA for modified M2e and amyloid β peptides was performed using methods described in our previous reports (15, 18).

Statistical analysis. Statistical analyses were performed using the Mann-Whitney U-test. A P value of <0.05 was considered statistically significant.

RESULTS

Adjuvant activity of citrulline to influenza virus HA
The influenza vaccine is popular worldwide. The influenza virus HA which forms multimeric rosette-like complexes is the antigen of the vaccine. Therefore, in this study, we used HA to determine the adjuvant activity of natural materials, one of which
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and forms virus-like particles. These inactivated virus and virus-like particles are well-known to have higher immunogenicity than that of proteins or peptides.

As shown in Fig. 2A and 2B, inactivated JEV and HBs with citrulline induced higher antibody titers although statistical differences were not significant between antigens with and without citrulline. The result indicates that citrulline has adjuvant activity against inactivated virus (JEV) and virus-like particles (HBs) but the activity is not strong compared to that shown in HA. As can be seen in the case of HBs, the differences in antibody titers between the presence and absence of citrulline were reduced by increasing the amount of HBs. It is considered that citrulline could not give a strong adjuvant effect to antigens which are intrinsically high immunogenic like inactivated JEV and HBs.

Adjuvant activities of citrulline to peptides

Antigens of vaccines are particulate, proteins, or peptides. Among them, peptides require adjuvants due to their weak immunogenicity. Previously, we reported the enhancement of peptide immunogenicity due to artificial binding of cysteine to the peptides. More specifically, the influenza virus M2e peptide and amyloid β peptide only upon the addition of cysteine residue(s) enhanced immunogenicity compared to that associated with the original peptides (15, 18). Therefore, we examined whether citrulline further increased the immunogenicity of these peptides. The mice were immunized with these cysteine-binding peptides (modified peptides) with or without citrulline, and antibody titers against

was citrulline. Mice were immunized using HA with or without citrulline. After immunization, antibody titers against HA were measured by ELISA. Immunization with more than 0.1 mg/head of citrulline induced higher antibody titers against HA than those achieved without citrulline (Fig. 1A). In this experiment, aluminum salt (Alum) revealed clear adjuvant activity against HA and 1 mg of citrulline induced antibodies comparable to aluminum salt.

In order to determine the optimal dose of citrulline, the mice were immunized with more than 1 mg/head of citrulline. Any dose of citrulline enhanced immunogenicity of HA to a greater extent than that in the group without citrulline, and 4 mg of citrulline showed the highest activity, although significant differences in activity were not observed between the various doses of citrulline (Fig. 1B). No side reactions such as abnormalities or skin induration at the site of injection were observed in the mice. Based on the result, the dose of 4 mg/head of citrulline was used for the subsequent studies.

Adjuvant activities of citrulline to inactivated JEV and HBs

Citrulline showed adjuvant activity to HA which is isolated from inactivated influenza virus and forms rosette-like complexes which are smaller than influenza virus. We then investigated the adjuvant activity of citrulline to other vaccine antigens, Japanese encephalitis and hepatitis B vaccine antigens.

The antigen of Japanese encephalitis vaccine is JEV inactivated with formaldehyde and has the same size as JEV. The hepatitis B vaccine consists of HBs which is produced by recombinant DNA technology and forms virus-like particles. These inactivated virus and virus-like particles are well-known to have higher immunogenicity than that of proteins or peptides.

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activity against various types of antigens, such as inactivated viruses, virus-like particles, and peptides.

**Difference in the adjuvant activity mechanism between citrulline and arginine**

Arginine, one of the intermediates of the urea cycle, is used as an immunostimulant in variety of human...
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According to guidelines of the European Medicines Agency (EMEA/CHMP/VEG/134716/2004), the actions of adjuvants are as follows: 1) presentation of the antigen, defined by the physical appearance of the antigen in the vaccine, 2) antigen/adjuvant uptake, 3) distribution (targeting to specific cells), 4) immune potentiation/modulation which includes activities that regulate both quantitative and qualitative aspects of the ensuing immune responses, and 5) the protection of the antigen from degradation and elimination.

Thus far, it is not known whether citrulline exerts its adjuvant activity through any of the abovementioned modes of action. However, this aspect needs to be elucidated in future studies. It is clear that citrulline would have an alternative mode of action compared to that of arginine while both amino acids constitute the urea cycle together. Unlike arginine, citrulline is known to have antioxidant activity (1). It was reported that some compounds with antioxidant activity have immunomodulatory effects although the modes of action remain unclear (7, 23, 27, 29). When modified M2e and amyloid β peptides were used as antigens, citrulline showed much higher adjuvant activity than that of the other vaccine antigens used in this study. These modified peptides have additional cysteine residues that might contribute toward increasing their immunogenicity. Therefore, the antioxidant character of citrulline is considered to amplify their immunogenicity.

DISCUSSION

It is known that citrulline has a variety of biological actions in human (2, 4, 5, 10, 14, 35). However, there have been no reports on its adjuvant activity until now. In this study, we first showed the adjuvant activity of citrulline to various types of antigens. Citrulline is highly soluble in aqueous solutions and commonly exists in bodies of mammals including humans. Furthermore, citrulline had no adverse reactions systemically and locally in the mice in our study. Therefore, these characteristics make citrulline a suitable adjuvant for vaccine formulations in clinical situations.

In this study, we first observed that citrulline has adjuvant activity, irrespective of the type of antigen. In some conditions, the effect of citrulline was much higher than that of the conventional aluminum salts. Furthermore, citrulline is thought to be safer than
aluminum salts because neither abnormality nor induration in topical areas was observed in mice in contradiction to aluminum salt injection which provoked inductions in topical areas.

Although the adjuvant activity of citrulline could be demonstrated in our study, its mechanism of action is not clear. Further investigations should involve confirmation of citrulline’s potential as an adjuvant candidate. Then, we hope that the new vaccines including this novel adjuvant with few side reactions and the high convenience will be launched in the near future.

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