Poly-γ-Glutamic Acid Nanoparticles and Aluminum Adjuvant Used as an Adjuvant with a Single Dose of Japanese Encephalitis Virus-Like Particles Provide Effective Protection from Japanese Encephalitis Virus

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To maintain immunity against Japanese encephalitis virus (JEV), a formalin-inactivated Japanese encephalitis (JE) vaccine should be administered several times. The repeated vaccination is not helpful in the case of a sudden outbreak of JEV or when urgent travel to a high-JEV-risk region is required; however, there are few single-injection JE vaccine options. In the present study, we investigated the efficacy of a single dose of a new effective JE virus-like particle preparation containing the JE envelope protein (JE-VLP). Although single administration with JE-VLP protected less than 50% of mice against lethal JEV infection, adding poly(γ-glutamic acid) nanoparticles (γ-PGA-NPs) or aluminum adjuvant (alum) to JE-VLP significantly protected more than 90% of the mice. A single injection of JE-VLP with either γ-PGA-NPs or alum induced a significantly greater anti-JEV neutralizing antibody titer than JE-VLP alone. The enhanced titers were maintained for more than 6 months, resulting in long-lasting protection of 90% of the immunized mice. Although the vaccine design needs further modification to reach 100% protection, a single dose of JE-VLP with γ-PGA-NPs may be a useful step in developing a next-generation vaccine to stop a JE outbreak or to immunize travelers or military personnel.

Japanese encephalitis virus (JEV) belongs to the genus Flavivirus, and it is distributed throughout India, China, Korea, Japan, and the countries of Southeast Asia. It has recently spread into new areas, including Australia (17–19, 29). JEV infection causes a severe central nervous system disease with symptoms of febrile headache, aseptic meningitis, and encephalitis; it has a high mortality rate (8, 10). Japanese encephalitis (JE) is the most important recognized cause of childhood viral encephalitis in Asia, with at least 50,000 clinical cases and 10,000 deaths every year (10). Currently, there is no treatment for this disease.

Vaccination is the only effective way to prevent JE in humans. In Japan, the Foundation for Microbial Diseases of Osaka University (BIKEN) manufactured a formalin-inactivated mouse brain-derived JE vaccine (JE VAC). However, several reports have indicated that its use is troubled by safety issues (22, 25). Thus, in 2009, BIKEN changed to manufacturing a Vero cell-derived JE vaccine. Recently, a second-generation JE vaccine, SA 14-14-2, which is a live attenuated vaccine, was licensed in China. Furthermore, the development of third-generation vaccines against JEV that do not involve live virus has also proceeded. For example, Kojima et al. (12, 21) produced a stable, high-producing cell line that makes JEV virus-like particles possessing the JEV envelope (E) protein (JE-VLP). Antibodies against E protein are typically neutralizing for JEV, and the authors demonstrated that two immunizations with JE-VLP were sufficient to protect mice from a lethal dose of JEV, given by injection.

In Japan, regular immunization with JE vaccine is performed according to an immunization law (11). First, the vaccine is given to children 3 times within their first 90 months (7.5 years of age), and a single booster injection is given between the ages of 9 and 12.
adjuvant (alum) is approved for hepatitis B virus (HBV) and human papillomavirus (HPV) vaccines in Europe, the United States, and Japan. In addition, liposomes are licensed for hepatitis A virus (HAV) and influenza vaccines in Europe (20).

Recently, Akagi et al. (1–3) generated biodegradable poly(γ-glutamic acid) (γ-PGA)-graft-1-phenylalanine (1-Phe) copolymers (γ-PGA nanoparticles [γ-PGA-NPs]). γ-PGA is a bacterial capsular exopolypolymer produced by certain strains of Bacillus subtilis (formerly Bacillus natto), which are natural components of a traditional Japanese food item, natto. γ-PGA-NPs are of natural origin, water soluble, and biodegradable (2), and they may be appropriate for medical use, with proposed applications as a drug delivery carrier, tissue-engineering material, and thermosensitive polymer (1–3). Furthermore, the particles are taken up by dendritic cells (DCs), resulting in the induction of cytokine production, the upregulation of costimulatory molecules, and the enhancement of their T-cell stimulatory capacity (34). We recently demonstrated that adding γ-PGA-NPs, as well as alum, to the influenza HA vaccine and to the JE vaccine enhances the protection they confer against their respective viruses (26, 27). Therefore, in the present study, we attempted to develop an effective single-dose immunization protocol by coadministering JE-VLP with γ-PGA-NPs or alum. We determined that specific humoral and cell-mediated immune responses for JEV were enhanced by the addition of adjuvants.

MATERIALS AND METHODS

Animals, virus strains, alum, and JE-VLP. Female BALB/c mice were obtained from Japan SLC, Inc. (Hamamatsu, Japan) and bred in our facility at the National Institute of Biomedical Innovation. All the mice used in this study were 4 weeks old. A JEV strain, Beijing 1 (the Kanonji Institute, the Research Foundation for Microbial Diseases of Osaka University [BIKEN], Kanonji, Japan), was used for the virus neutralization assay, and JaTH-160 (BIKEN) was used for the mouse challenge experiments. JE-VLP was prepared by BIKEN as described by Kojima et al. (12). The alum for vaccinations and the JE vaccine (BIKEN), a formalin-inactivated JEV VLP was prepared by BIKEN as described by Kojima et al. (12). The alum for vaccinations and the JE vaccine (BIKEN), a formalin-inactivated JEV produced in mouse brain (JE-VLP), were prepared by BIKEN. The lot numbers of the mouse vaccine BIKEN we used in this study were JEO31 and JEO32.

Synthesis and preparation of γ-PGA-NPs. γ-PGA (Mn = 380,000) was kindly donated by Meiji Seika Co., Ltd. (Tokyo, Japan). Nanoparticles composed of γ-PGA hydrophobic derivatives were prepared as previously described (2, 26). Briefly, γ-PGA was hydrophobically modified by 1-phenylalanine ethyl ester (1-Phe) (Sigma, St. Louis, MO) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (WSC; Wako Pure Chemical Industries, Osaka, Japan). The purified γ-PGA-graft-1-Phe was characterized by H nuclear magnetic resonance (1H-NMR) to determine the degree of 1-Phe grafting. In this experiment, γ-PGA-graft-1-Phe with a 53% grafting degree was used. Nanoparticles composed of γ-PGA-graft-1-Phe were prepared by a precipitation and dialysis method. First, the γ-PGA-graft-1-Phe (10 mg) was dissolved in 1 ml dimethyl sulfoxide (DMSO), and saline at the same volume was added to yield a translucent solution. The solutions were then dialyzed against distilled water using cellulose membrane tubing (50,000 molecular weight cutoff) to remove the organic solvents. The dialyzed solutions obtained were then freeze-dried.

Vaccination. Vaccination was performed according to a method described previously (12, 23). BALB/c mice (4 weeks old, female) were given an intraperitoneal injection of 400 µL PBS or 400 µL phosphate-buffered saline (PBS) containing 1 µg JE-VLP, 1 µg JE VAC, 1 µg JE-VLP with 100 µg alum, 1 µg JE VAC with 100 µg alum, 1 µg JE-VLP with 100 µg γ-PGA-NPs, or 1 µg JE VAC with 100 µg γ-PGA-NPs. Some groups of mice were given a second injection 7 days later.

Test for neutralizing antibodies in serum samples. Serum samples from the single-dose-immunized mice were harvested 15 days after the first immunization, and each sample was tested for virus-neutralizing antibodies, as described previously (12, 30). Briefly, serial 4-fold dilutions of serum samples pretreated with heat at 56°C for 30 min were incubated with 100 PFU of the JEV strain Beijing 1 in minimal essential medium (MEM) supplemented with 2% fetal bovine serum (FBS) for 1.5 h at 37°C. A 200-µl aliquot was examined for residual virus infectivity by plaque assay on Vero cell monolayers in 6-well plates. The percent plaque reduction was calculated relative to virus controls incubated without mouse serum. Neutralizing antibody titers were expressed as the reciprocal of the serum dilution yielding a 50% reduction in the mean plaque number versus that of control wells.

Virus challenge. For the virus challenge, groups of immunized mice were given intraperitoneal injections of 3 × 106 PFU (20 times the 50% lethal dose [LD50]) of the JaTH-160 strain of JEV on day 15 after the first vaccination. Simultaneously with the JEV challenge, mice were given 10 µL PBS by intracerebral injection to disrupt the blood-brain barrier, as described previously (12, 30). The challenged mice were observed daily for 20 days.

Statistical evaluations. Fisher’s exact test was performed using Statcel2 software (OMS, Tokyo, Japan) to evaluate the differences between groups in the mortality experiments. To analyze the data in the other experiments, the parametric Welch t test was used. P values of <0.05 were considered significant.

RESULTS

Efficacy of protection against a lethal dose of JEV by a single immunization with JE-VLP alone. We first examined whether one or two immunizations with JE-VLP induced sufficient protection for mice to survive a lethal JEV infection. Groups of mice were immunized twice with 1 µg JE VAC or JE-VLP at 7-day intervals. On day 15 after the primary immunization, the mice were infected with JEV strain JaTH-160 and their mortality rate was assessed. All mice (10/10) or 90% of the mice (9/10) that received two injections of JE VAC or JE-VLP survived the lethal JEV infection (Fig. 1A). In contrast, only 50% (5/10) or 40% (4/10) of the mice given a single injection of JE VAC or JE-VLP survived (Fig. 1B). Next, serum samples collected 15 days after the primary immunization and vaccination regimen were used in a plaque reduction assay. As shown in Fig. 1C, the neutralizing antibody titers in the sera of mice immunized once with JE VAC or JE-VLP were significantly lower than those of the twice-immunized mice.

Effect of adjuvants on protective immune responses induced by a single JE-VLP injection against lethal JEV infection. Next, we determined whether γ-PGA-NPs and alum were effective adjuvants for the single-dose JE-VLP strategy. Groups of mice were immunized once with JE-VLP alone or with a mixture of JE-VLP and γ-PGA-NPs, and the mice were infected with JEV 15 days after the final immunization. The mortality assessment showed that single-dose immunization with the mixture of JE-VLP and γ-PGA-NPs markedly enhanced survival. Twenty days after the JEV infection, 93.9% (31/33) of the mice that had been given a single dose of JE-VLP with γ-PGA-NPs were still alive, whereas only 41.9% (13/31) of the mice given only a single injection of JE-VLP survived the infection (Fig. 2). In addition, all mice (20/20) that had been given a single dose of JE VAC with γ-PGA-NPs were still alive, whereas only 40.0% (10/20) of the mice given only a single injection of JE VAC survived the infection (Fig. 2). At the same time, groups of mice were immunized once with a mixture of 1 µg JE-VLP and 100 µg alum. The mice were infected with JEV, and their mortality rate was assessed. Twenty days after...
the JEV infection, 93.9% (31/33) of these mice were still alive (Fig. 2). In addition, all mice (20/20) that had been given a single dose of JE VAC with alum were still alive (Fig. 2).

Enhancement of JEV antigen-specific humoral immune responses by the administration of JE-VLP with adjuvants. We determined whether the JEV antigen-specific humoral immune responses were increased by the addition of adjuvant to the JE VAC or JE-VLP. Mice were immunized intraperitoneally once, and the levels of anti-JEV neutralizing antibodies in their serum were assessed. Immunization with the mixture of JE VAC or JE-VLP and γ-PGA-NPs induced significantly higher titers of anti-JEV neutralizing antibodies than did the immunization with JE VAC or JE-VLP alone (Fig. 3). Adding alum instead of γ-PGA-NPs adjuvants on JE-VLP protection against death from JEV infection. Groups of mice were immunized by intraperitoneal injection of 400 µl PBS (black circles; n = 31), 400 µl PBS containing 100 µg alum (black squares; n = 35), 100 µg γ-PGA-NPs (black diamonds; n = 35), 1 µg JE-VLP (red circles; n = 31), 1 µg JE-VLP and 100 µg alum (red squares; n = 33), 1 µg JE-VLP and 100 µg γ-PGA-NPs (red diamonds; n = 33), 1 µg JE VAC (blue circles; n = 20), 1 µg JE VAC and 100 µg alum (blue squares; n = 20), or 1 µg JE VAC and 100 µg γ-PGA-NPs (blue diamonds; n = 33) on day 0. On day 15, the mice were infected with JEV and survival was assessed daily for 20 days after the infection. #, P < 0.05 versus mice immunized with JE-VLP; **, P < 0.05 versus mice immunized with JE VAC.

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NPs to the immunization also enhanced the anti-JEV neutralizing antibody titer (Fig. 3). However, the neutralizing antibody titers in mice immunized with JE-VLP plus adjuvants were lower than in those immunized with JE VAC plus adjuvants (Fig. 3).

**Effect of adjuvants on length of protection induced by a single JE-VLP injection against lethal JEV infection.** We found that neutralizing antibody titers in the serum samples from mice immunized with JE VAC or JE-VLP mixed with either alum or γ-PGA-NPs were maintained for 6 months, and they remained significantly higher than the titers induced with JE VAC or JE-VLP alone, over long-term observation (Fig. 4). Therefore, we next determined whether the long-lasting neutralizing antibodies afforded long-lasting protection. Groups of mice were immunized once with JE-VLP alone or with a mixture of JE-VLP and γ-PGA-NPs, and the mice were infected with JEV 6 months after the final immunization. The mortality assessment showed that single-dose immunization with JE-VLP mixed with either γ-PGA-NPs or alum markedly enhanced survival, with a rate of 90% (9/10) in both cases, whereas only 20% (2/10) of mice given a single injection of JE-VLP alone survived the infection (Fig. 5). The mortality assessment showed that single-dose immunization with the mixture of JE VAC and either γ-PGA-NPs or alum markedly enhanced survival to 100% (10/10) in both immunizations, whereas only 20% (2/10) of the mice given a single injection of JE VAC alone survived the infection (Fig. 5).

**DISCUSSION**

A recent study showed that the 53-kDa envelope (E) glycoprotein of JEV has an important role in virus adhesion and entry into target cells through receptor binding and that a neutralizing antibody for the E glycoprotein protects hosts against JEV infection (5). Molecular biological studies of JEV have shown that the expression of the premembrane (prM) and E genes in mammalian cells leads to the production of small, capsid-less, noninfectious JE-VLP that possess the E antigen (13–15). Furthermore, Kojima et al. (12, 21) made a stable, high-producing clonal cell line that produces JE-VLP that includes the E protein; these authors demonstrated that two injections of JE-VLP effectively protects mice from JEV infection. In the present study, a single immunization with JE-VLP alone induced a serum titer of anti-JEV neutralizing antibodies that was one-tenth of that induced by two injections of JE-VLP.
JE-VLP alone and protected only 40% of the mice from a lethal dose of JEV. However, using an adjuvant, either the current clinical standard, alum, or the new adjuvant candidate, γ-PGA-NPs, with the JE-VLP enhanced the anti-JEV neutralizing antibody titer, which resulted in the survival of more than 90% of the mice. The results suggest that adding alum or γ-PGA-NPs to the single JE-VLP vaccination enhances protection; this might be due to enhanced anti-JEV neutralizing antibody secretion. It should be noted that adding alum or γ-PGA-NPs to JE-VLP or JE VAC not only enhanced protection but also induced a long-lasting production of neutralizing antibodies. However, it is not clear how these adjuvants induced the long-lasting neutralizing antibody production and long-lasting protection from a single JE-VLP or JE VAC immunization.

We previously demonstrated that immunization with a single injection of JE VAC with adjuvants protected all immunized mice against lethal JEV infection (27). On the other hand, immunization with a single injection of JE-VLP with adjuvants protected 90%, but not 100%, of mice against JEV infection. The means of neutralizing antibody titers in mice immunized with JE VAC plus adjuvant were more than 10^4.5 ± 10^4.49 and 10^6.04 ± 10^6.26 for JE VAC plus alum and JE VAC plus γ-PGA-NPs, respectively). However, the titers in mice immunized with JE-VLP plus adjuvants were lower than in those with JE VAC plus adjuvant (10^4.41 ± 10^4.53 and 10^2.45 ± 10^9.48 for JE-VLP with alum and JE-VLP with γ-PGA-NPs, respectively). Furthermore, the neutralizing antibody titer of one mouse immunized with JE-VLP and alum and one with JE-VLP and γ-PGA-NPs was less than 10^2 (JE-VLP with alum, 10^0.78; JE-VLP with γ-PGA-NPs, 10^1.68) (Fig. 3). On the other hand, the titers from a single immunization with JE-VLP or JE VAC were almost all 10^2 (10^0.55 ± 10^0.39 and 10^3.56 ± 10^3.31, respectively) (Fig. 3), and a JEV infection survival rate was less than 50% (Fig. 1 to 3). The results suggest that a neutralizing antibody titer less than 10^2 cannot ensure 100% protection. This may explain why 100% of the mice immunized with JE VAC plus adjuvant, but only 90% of those immunized with JE-VLP plus adjuvant, were protected. However, the source of the difference in the protective immune responses between JE VAC and JE-VLP has not been revealed and will require further investigation.

It remains to be learned how the mixture of JE-VLP and γ-PGA-NPs enhanced the JEV antigen-specific humoral immunity. γ-PGA-NPs are nanoparticles with a diameter of 200 nm, and various molecules, including vaccine antigens, can be immobilized on their surfaces or packaged inside them (1). The particles can deliver drugs into dendritic cells (DCs) and direct the activation of DCs via enhanced Toll-like receptor 4 (TLR4) and the MyD88 signaling pathway (32, 33). Furthermore, the γ-PGA-NPs themselves were taken up by DCs and sequestered in the lysosomal compartments, which resulted in an enhanced ability of the DCs to stimulate T cells (34). Therefore, there is some synergy between the role of γ-PGA-NPs in antigen delivery and their role in DC activation that is important for enhancing antigen-specific immune responses.

Alum is the vaccine adjuvant approved worldwide. Despite its having been injected into billions of people, its mechanism of action is not fully understood. Recent studies show that the nucleotide-binding oligomerization domain-containing protein (NOD)-like receptor, the pyrin domain containing 3 (Nlrp3) inflammasome, is the molecular target of alum immunostimulatory activity (6, 7, 9). However, it still unclear whether the Nlrp3 inflammasome is dispensable for alum’s adjuvant activity (7, 9). Eisenbarth et al. (7) showed that, in vitro, alum induced the direct activation of the Nlrp3 inflammasome complex and synergized with the LPS stimulation of TLR4 to promote the secretion of proinflammatory cytokines, such as interleukin-1β (IL-1β), IL-18, and IL-33. In vivo, alum induces necrosis in unidentified target cells, resulting in the production of uric acid, which has the potential to stimulate Nlrp3 (16). Although it remains to be learned how the mixture of alum with JE-VLP induces antigen-specific humoral and cell-mediated immune responses, synergy may be induced by a novel signaling cascade, different from the one that mediates the synergy between alum and LPS. Therefore, investigations to determine the existence of such a novel signaling cascade should be undertaken.

γ-PGA-NPs are of natural origin, water soluble, and biodegradable (2), and γ-PGA is an edible and natural biopolymer (28, 31). The subcutaneous injection of 10 mg/mouse γ-PGA-NPs did not induce tissue injury or an infiltration of inflammatory cells, nor did it induce any sign of acute toxicity (35). Furthermore, when the γ-PGA-NPs alone were administered using the two-dose protocol, the mice did not mount an anti-γ-PGA-NPs immune response (unpublished data). In summary, this study suggests that a single dose of JE-VLP mixed with either γ-PGA-NPs or alum enhanced JE-specific neutralizing antibody protection and protection against the virus. This combination may provide the basis for designing a third-generation JE vaccine for travelers and military personnel.

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