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Contents

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This issue contains three papers from the 3rd Annual Conference on Vaccine Research 30 April-3 May 2000

Editorial

- 4339 FMD in the UK—the 2001 outbreak; what if...?
R. E. Spier

Conference Reports

- 4342 Report on *The Third Annual Walker's Cay Colloquium on Cancer Vaccines*
A. L. Goldstein
- 4347 Meningococcal disease in Europe: epidemiology, mortality, and prevention with conjugate vaccines. Report of a European advisory board meeting Vienna, Austria, 6-8 October, 2000
K. Cartwright, N. Noah, H. Peltola and On behalf of the Meningococcal Disease Advisory Board

Letter to the Editor

- 4357 Antineoplastic therapy in children and adolescents – a retrospective analysis
S. C. Arya

Short Communication

- 4358 Endemic infection of a cat colony with a feline calicivirus closely related to an isolate used in live attenuated vaccines
A. D. Radford, L. Sommerville, R. Ryvar, M. B. Cox, D. R. Johnson, S. Dawson and R. M. Gaskell

Review

- 4363 Towards the rational design of Th1 adjuvants
P. Moingeon, J. Haensler and A. Lindberg

3rd Annual Conference on Vaccine Research

- 4373 Live attenuated influenza virus vaccines: new options for the prevention of influenza
K. L. Nichol
- 4378 Progress towards global polio eradication
H. F. Hull and R. B. Aylward
- 4385 From genome to vaccine: in silico predictions, ex vivo verification
A. S. De Groot, A. Bosma, N. Chinai, J. Frost, B. M. Jesdale, M. A. Gonzalez, W. Martin and C. Saint-Aubin

[continued overleaf]



The Official Journal of the International Society for Vaccines

CONTENTS
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Regular Papers

- 4396 Immunogenicity and reactogenicity of diphtheria, tetanus and pertussis toxoids combined with inactivated polio vaccine, when administered concomitantly with or as a diluent for a Hib conjugate vaccine
N. Knutsson, B. Trollfors, J. Taranger, E. Bergfors, V. Sundh, T. Lagergård, E. Östergaard, H. Cicirello and H. Käyhty
- 4404 IgG antibody subclass responses determined by immunoblot in infants' sera following vaccination with a meningococcal recombinant hexavalent PorA OMV vaccine
S. Martin, F. Sadler, R. Borrow, M. Dawson, A. Fox and K. Cartwright
- 4409 Passive transfer of protection against *Bacillus anthracis* infection in a murine model
R. J. Beedham, P. C. B. Turnbull and E. D. Williamson
- 4417 The comparative efficacy of CTLA-4 and L-selectin targeted DNA vaccines in mice and sheep
D. R. Drew, J. S. Boyle, A. M. Lew, M. W. Lightowers, P. J. Chaplin and R. A. Strugnell
- 4429 A randomised comparison of two inactivated hepatitis A vaccines, Avaxim™ and Vaqta™, given as a booster to subjects primed with Avaxim™
P. Clarke, N. Kitchin and F. Souverbie
- 4434 Protection against influenza virus challenge by topical application of influenza DNA vaccine
S. Watabe, K.-Q. Xin, A. Ihata, L.-J. Liu, A. Honsho, I. Aoki, K. Hamajima, B. Wahren and K. Okuda
- 4445 Biodegradable PLGA microspheres as a delivery system for malaria synthetic peptide SPf66
J. E. Rosas, R. M. Hernández, A. R. Gascón, M. Igartua, F. Guzman, M. E. Patarroyo and J. L. Pedraz
- 4452 Antigenic and molecular heterogeneity in recent swine influenza A(H1N1) virus isolates with possible implications for vaccination policy
J. C. de Jong, P. P. Heinen, W. L. A. Loeffen, A. P. van Nieuwstadt, E. C. J. Claas, T. M. Bestebroer, K. Bijlsma, C. Verweij, A. D. M. E. Osterhaus, G. F. Rimmelzwaan, R. A. M. Fouchier and T. G. Kimman
- 4465 Role of antibody to lipopolysaccharide in protection against low- and high-virulence strains of *Francisella tularensis*
M. Fulop, P. Mastroeni, M. Green and R. W. Titball
- 4473 Immunogenicity and efficacy of one dose measles-mumps-rubella (MMR) vaccine at twelve months of age as compared to monovalent measles vaccination at nine months followed by MMR revaccination at fifteen months of age
M. Ceyhan, G. Kanra, G. Erdem and B. Kanra
- 4479 Efficacy of vaccination of pigs with different H1N1 swine influenza viruses using a recent challenge strain and different parameters of protection
K. Van Reeth, G. Labarque, S. De Clercq and M. Pensaert
- 4487 *Plasmodium falciparum* circumsporozoite (CS) protein peptides specifically bind to HepG2 cells
J. E. Suarez, M. Urquiza, A. Puentes, J. E. Garcia, H. Curtidor, M. Ocampo, R. Lopez, L. E. Rodriguez, R. Vera, M. Cubillos, M. H. Torres and M. E. Patarroyo
- 4496 *Plasmodium falciparum* acid basic repeat antigen (ABRA) peptides: erythrocyte binding and biological activity
H. Curtidor, M. Urquiza, J. E. Suarez, L. E. Rodriguez, M. Ocampo, A. Puentes, J. E. Garcia, R. Vera, R. Lopez, L. E. Ramirez, M. Pinzon and M. E. Patarroyo
- 4505 Potential of *Klebsiella pneumoniae* cytotoxin toxoid as vaccine against klebsiellosis in rabbits and mice
B. R. Singh and V. D. Sharma
- 4511 Rapid clearance of SAG-2 rabies virus from dogs after oral vaccination
L. A. Orciari, M. Niezgoda, C. A. Hanlon, J. H. Shaddock, D. W. Sanderlin, P. A. Yager and C. E. Rupprecht
- 4519 Nasal absorption and biodistribution of plasmid DNA: an alternative route of DNA vaccine delivery
Y.-K. Oh, J.-P. Kim, T. S. Hwang, J. J. Ko, J. M. Kim, J.-S. Yang and C.-K. Kim
- 4526 DNA vaccination against v-src oncogene-induced tumours in congenic chickens
J. Plachý, J. Hejnar, K. Trtková, K. Trejbalová, J. Svoboda and K. Hála
- 4536 First field trial of a transmissible recombinant vaccine against myxomatosis and rabbit hemorrhagic disease
J. M. Torres, C. Sánchez, M. A. Ramírez, M. Morales, J. Bárcena, J. Ferrer, E. Espuña, A. Pagès-Manté and J. M. Sánchez-Vizcaíno
- 4544 High seroprotection rate induced by low doses of a recombinant hepatitis B vaccine in healthy Iranian neonates
F. Shokri and A. Jafarzadeh
- 4549 Features of the antibody response attributable to plasmid backbone adjuvanticity after DNA immunization
M. Hervé, L. Dupré, E. Ban, A.-M. Schacht, A. Capron and G. Riveau
- 4557 A purified inactivated Japanese encephalitis virus vaccine made in vero cells
A. K. Srivastava, J. R. Putnak, S. H. Lee, S. P. Hong, S. B. Moon, D. A. Barvir, B. Zhao, R. A. Olson, S.-O. Kim, W.-D. Yoo, A. C. Towle, D. W. Vaughn, B. L. Innis and K. H. Eckels
- 4566 Surveillance for meningococcal disease and strategies for use of conjugate meningococcal vaccines in the United States
J. R. Lingappa, N. Rosenstein, E. R. Zell, K. A. Shutt, A. Schuchat, B. A. Perkins and Active Bacterial Core Surveillance (ABCs) Team
- 4576 **Meeting Announcement**
- 4577 **Announcement: 3rd World Congress on Vaccines and Immunisation**

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A purified inactivated Japanese encephalitis virus vaccine made in vero cells

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Abstract

A second generation, purified, inactivated vaccine (PIV) against Japanese encephalitis (JE) virus was produced and tested in mice where it was found to be highly immunogenic and protective. The JE-PIV was made from an attenuated strain of JE virus propagated in certified Vero cells, purified, and inactivated with formalin. Its manufacture followed current GMP guidelines for the production of biologicals. The manufacturing process was efficient in generating a high yield of virus, essentially free of contaminating host cell proteins and nucleic acids. The PIV was formulated with aluminum hydroxide and administered to mice by subcutaneous inoculation. Vaccinated animals developed high-titered JE virus neutralizing antibodies in a dose dependant fashion after two injections. The vaccine protected mice against morbidity and mortality after challenge with live, virulent, JE virus. Compared with the existing licensed mouse brain-derived vaccine, JE-Vax, the Vero cell-derived JE-PIV was more immunogenic and as effective as preventing encephalitis in mice. The JE-PIV is currently being tested for safety and immunogenicity in volunteers. © 2001 Published by Elsevier Science Ltd.

Keywords: JE-PIV; Vero cells; Vaccine

1. Introduction

Japanese encephalitis virus (JEV) is a member of the flavivirus family and the causal agent of Japanese encephalitis (JE). It is a small (50 nm), enveloped virus

containing a 10.7 kb, single-stranded, RNA genome, the complete sequence of which has been determined [1]. Like other flaviviruses, the virion RNA serves as message for 11 proteins: four virus structural proteins, capsid (C), premembrane (prM), membrane (M) and envelope (E), and seven nonstructural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 [2]. The viral E protein, modified by glycosylation and dimerization during virion assembly, serves as the cell-receptor binding protein and the fusion protein for virus attachment and entry into the host. Antibodies directed against E protein neutralize the virus [3] and play an important role in protection [4].

JEV remains a major public health threat. Although it is not as widely distributed as the dengue viruses, it is common in large parts of Asia, India and the Western

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Pacific [5–7]. Like dengue, the virus is transmitted to its hosts, i.e. humans, birds and swine, by mosquitoes. After infection, the non-human hosts often develop high levels of viremia, without apparent disease; thus, they act as natural reservoirs for the virus [8]. In humans, where levels of viremia are generally low and no well-defined clinical symptoms are usually seen, only a small percentage of infected individuals will develop encephalitis [5]. However, in those individuals the mortality rate may exceed 30% and many will suffer serious, long-term, neurologic effects [5,9].

The only reliable way of preventing JE is by vaccinating at-risk populations. Currently, there is only one JE vaccine licensed by the United States Food and Drug Administration USFDA for human use. This vaccine, manufactured by the BIKEN Institute in Japan and distributed commercially in the United States by Aventis-Pasteur under the trade name JE-Vax, is made from a virulent strain of JEV, propagated in suckling mouse brains, purified, and inactivated with formalin [10]. In a large field trial in Thailand, the vaccine was shown to be highly effective at preventing encephalitis [11]. Although the safety record of JE vaccine in use in Japan has been excellent, local reactions such as tenderness, redness and swelling have been reported in up to 20% of American, European and Australian vaccinees, and mild systemic side effects such as fever, chills, headache and malaise have been reported in ~10%. Since 1986, there have been more reports of allergic reactions, which take the form of itching, urticaria and occasionally angioedema of the face with difficulty in breathing, sometimes requiring hospitalization and corticosteroid therapy [12–14]. In about one-third of these cases, however, the patients appeared to have a predisposition to allergic reactions, although specific allergens have not been identified in the vaccine. It is not known if these problems are related to certain lots of the vaccine or to its use in different populations.

The only other JE vaccine currently in widespread use is SA₁₄-14-2, a live attenuated virus vaccine (LAV) produced in the Peoples Republic of China [15,16]. This vaccine was derived from a wild virus isolate, SA₁₄, by attenuation in primary hamster kidney cells, mice and hamsters [15]. It appears to be safe and effective at preventing encephalitis [16,17]; however, concern about possible adventitious agents in primary hamster cells will likely limit its use outside of China. Earlier, an attempt was made to produce the Chinese LAV in a better-characterized cell substrate by adapting SA₁₄-14-2 virus to primary dog kidney (PDK) cells, which are certified for vaccine production. This candidate vaccine, designated SA₁₄-14-2, PDK-9, was sequenced and found to have accumulated some additional nucleotide changes when

compared with SA₁₄-14-2 [18]. However, none of these changes appear to represent reversion to wild-type. Although SA₁₄-14-2, PDK-9, passed its initial evaluation for safety and immunogenicity in volunteers, it was not developed further.

The goal of the present work was to produce a second generation JE vaccine in Vero (*Cercopithecus aethiops*) cells that is safe, effective, and possibly more economical than the existing licensed vaccine. There is a precedent for using Vero cells for making vaccines against polio [19] and rabies [20,21], as well as an experimental purified, inactivated vaccine (PIV) against dengue type-2 virus [22]. The JE-PIV described in the present report was produced using methods originally developed for making the dengue-2 PIV [22]. SA₁₄-14-2, PDK-8 production seed virus was serially passaged in a certified line of Vero cells to make a new vaccine production seed, which was then grown in Vero cells, purified, and inactivated with formalin. The JE-PIV was tested in mice where it was found to be highly immunogenic and protective. It is currently undergoing testing for safety and immunogenicity in volunteers and will shortly undergo testing for protective efficacy in non-human primates.

2. Materials and methods

2.1. Cells and cell propagation

Vero African green monkey (*Cercopithecus aethiops*) kidney cells [23,24] were used to propagate the vaccine virus. These cells were obtained from a certified master cell bank maintained at the Salk Institute Government Services Division, Swiftwater, PA, at cell passage level-139. The identity of the cells was established by karyotyping and isoenzyme analysis. They were also tested and found to be negative for adventitious agents, small animal tumorigenicity, and reverse transcriptase activity. Twelve 1 ml frozen ampoules containing ~10 million cells each were seeded into 36 150-cm² plastic (T150) flasks. The flasks were fed with 50 ml of growth medium (GM) containing Eagles Minimal Essential Medium (EMEM), 1.1 g sodium bicarbonate/l, 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 100 units each of streptomycin and neomycin (S/N). Flasks were incubated tightly capped in a 35 °C incubator. Under these conditions the monolayers were confluent within 5 days. Confluent cell monolayers were trypsinized and subcultured into 490 cm² roller bottles, one flask per roller bottle. Roller bottle cultures were fed with 100 ml of GM and incubated at 35 °C on a roller apparatus rotating at 1 rpm. After 4 days the cells were confluent and ready for virus inoculation.

2.2. Viruses and vaccines

Attenuated JE vaccine SA₁₄-14-2 was previously passaged eight times in certified primary dog kidney (PDK) cells [18]. The PDK cell-passaged virus was then passaged five times in certified Vero cells and used as the production seed for making the inactivated vaccine. The USFDA licensed, inactivated, JE vaccine produced by BIKEN, Japan, and distributed as JE Vax in the US, was purchased from Aventis-Pasteur.

2.3. Antisera

The antigenic specificity of the vaccine virus was determined using anti-JE virus-specific mouse hyperimmune ascitic fluid (MHAF), M1/311, which was prepared as previously described [25]. This antiserum was also used to neutralize JE virus in the harvested culture fluids for adventitious agent testing.

2.4. Virus production

Confluent Vero cell monolayers in 490 cm² roller bottles were washed three times with Dulbecco's phosphate buffered saline containing 2 mM calcium and 1 mM magnesium chloride (DPBS) and then infected with JEV SA₁₄-14-2, PDK-8, Vero-5. Infection was performed at a multiplicity of ~0.01 pfu/cell with the inoculum contained in 5 ml of maintenance medium (MM) containing EMEM, 2.2 g sodium bicarbonate/l, 2% FBS, 2 mM L-glutamine, and S/N. After infection, the cultures were replaced on a roller apparatus (1 rpm) and incubated at 35 °C for 1 h to allow for virus adsorption. After adsorption the cultures were again washed three times with 100 ml of DPBS, fed with 100 ml of serum-free MM and placed on a roller apparatus (1 rpm) at 35 °C. A complete harvest of the culture media was made on days 3, 5, 7, and 9 post-inoculation and each time the cultures were refed with 100 ml of serum-free medium.

2.5. Virus purification

Pooled culture media from the virus harvests was clarified by centrifugation (1000 × g, 10 min, 4 °C). The cell pellets were discarded and the supernatants were filtered through a 0.45 µm nitrocellulose filter unit. The resulting filtrate (~12 l) was applied to a 100000 molecular weight cut-off (MWCO), tangential flow, ultrafiltration unit (Filtron, Omega 100 K Screen Channel). It was concentrated to ~350 ml at a pressure, delivered by vacuum pump, of 20–30 psi, a cross-flow rate of 400 ml/min, and a filtration rate of ~100 ml/min. The virus concentrate was then treated with protamine sulfate at a final concentration of 2 mg/ml. The virus-protamine mixture was incubated at 4 °C on

wet ice for 2 h. The protamine precipitate was pelleted in a TY30 rotor at 17000 rpm and 4 °C for 5 min, and the virus-containing supernatant was transferred to a clean, sterile container and stored temporarily at 4 °C. The virus was purified on discontinuous sucrose gradients prepared as follows: Centrifuge tubes (Beckman Ultraclear™, 1.5 × 3.5 in.) were sanitized by addition of 10% H₂O₂ for 30 min at room temperature, the H₂O₂ was decanted and the tubes rinsed three times with sterile PBS. Sucrose gradients were prepared by stepwise addition of 60, 55, 50, 45, 40, 35, and 30 (2 ml each) and 15% (1 ml) w/w sucrose in PBS. The gradients were allowed to diffuse at 20 °C for 2–5 h, then 25 ml of virus concentrate was carefully layered onto each gradient and the gradients were centrifuged in a Beckman SW28 rotor at 17000 rpm and 4 °C for 18 h. One ml fractions were collected from the bottom of the tube using a positive displacement fraction collector that introduces light mineral oil into the top of the tube. The fractions were assayed for JEV by hemagglutination (HA) of goose RBCs as previously described [22]. Fractions with peak HA activity (> 1:3200) and those with up to eight-fold lower activity (> 1:400) were pooled into a sterile 500 ml flask (Nalgene). The virus pool was diluted approximately eight-fold with PBS, pH 7.4 and filtered through a 0.22 µm low protein binding GV-type filter (Millipore), then stored temporarily at 4 °C.

2.6. Formalin inactivation

Formalin (37% formaldehyde, Sigma, St. Louis, MO) was diluted 1:40 in PBS (the final pH 7.2–7.4, adjusted with 0.1 N NaOH if necessary). The solution was filtered through a 0.22 µm nitrocellulose filter unit. One part of the 1:40 formalin solution was added dropwise to 50 parts of the diluted, filtered virus pool for a final formalin dilution of 1:2000 (concentration of 0.05%, ~5 mM). Virus inactivation was carried out at 22 °C in a constant temperature, circulating water bath. One ml samples each for residual live virus assay and antigen assay were taken from the virus pool at daily intervals. The formalin in the samples was neutralized with sodium bisulfite by addition of one part 3.75% (w/v) sodium metabisulfite to 100 parts of vaccine. Forty-eight h after addition of formalin, the virus pool was again filtered through a 0.22 micron low protein binding (GV-type) filter unit, transferred to a clean, sterile container, and inactivation was resumed for a total of 240 h. Following the inactivation period, the residual formalin in the bulk vaccine was neutralized with sodium bisulfite as described above. The neutralized bulk vaccine was placed in sterile dialysis tubing (Sepcor 7, 25 mm, Spectrum) and dialyzed at 4 °C against ~50 volumes of PBS, pH 7.4, which was changed twice. Following dialysis, the vaccine was

again filtered through a 0.22 micron low protein-binding (GV-type) filter unit, and stored at 4 °C in a clean, sterile 500 ml container (Nalgene), designated as vaccine bulk lot 0475.

2.7. Final vaccine formulation and bottling

Three hundred ml of the dialyzed bulk vaccine was transferred aseptically to a clean, sterile, 500 ml bottle (Nalgene). Fifteen ml of aluminum hydroxide (Rehydralgel 2% aluminum hydroxide, Reheis) was added to the 300 ml of vaccine. The solution was manually swirled then set on an orbital shaker set at 90 rpm for 1 h 25 min at room temperature. A solution of Thimerisol was prepared by addition of 1 g Thimerisol powder to 100 ml of sterile PBS. After the Thimerisol was dissolved the solution was filtered through a 0.22 µm nitrocellulose filter unit (Nalgene). A total of 3.15 ml of the 1% Thimerisol solution was added to the vaccine, which was left to mix on the shaker for an additional 10 min. The formulated vaccine was then aliquoted aseptically, 4 ml per vial, into sterile 5 ml glass serum tubing vials (Wheaton). The filled vials were stoppered, crimped and stored at 4 °C. This vaccine was designated as lot 0574.

2.8. Polyacrylamide gel electrophoresis, Coomassie blue staining, and Western blotting

20 ml samples of JE-PIV, lot 0475, were boiled for 3 min in a buffer containing Tris, pH 7.2, 1% sodium dodecylsulfate (SDS), 0.1 M 2-mercaptoethanol, and 0.5% bromophenol blue, BPB. They were applied to 0.75 mm thick, 7.5–20%, discontinuous polyacrylamide gels containing 1% SDS and electrophoresed at constant current (25 mA) until the BPB dye front reached the bottom of the gel. Following electrophoresis, one gel was fixed in 30% methanol and 10% acetic acid and stained with 1% Coomassie brilliant blue-R to visualize the resolved protein bands. Resolved proteins from an unstained gel were transferred electrophoretically to nitrocellulose paper and detected by a modification of Western blotting [26]. The nitrocellulose was incubated for 30 min at room temperature with 5% powdered, skimmed milk, then washed with phosphate buffered saline (PBS), pH 7.4, and incubated overnight at 4 °C with anti-JE hyperimmune mouse ascitic fluid, M1/311, diluted 1:250 in PBS. After this incubation, the nitrocellulose was washed three times with PBS and incubated for 4 h at room temperature with a 1:500 dilution of goat anti-mouse antibody conjugated to horseradish peroxidase (HRP). After this incubation the nitrocellulose was washed three times in PBS and incubated with a chromagenic HRP substrate in order to visualize the bound antibody.

2.9. Virus plaque reduction neutralization assay

JEV neutralizing antibody in mouse sera was measured using a virus plaque-reduction neutralization test (PRNT) with a 50% plaque reduction endpoint (PRNT₅₀). This assay was performed as previously described [19], except that Vero cells were used rather than LLC-MK₂ cells.

2.10. Virus hemagglutination (HA) assay

The HA assay was performed by the method of Clarke and Casals [27], adapted to 96-well microtiter plate-format.

2.11. Protein assay

Protein assay was performed using the BioRad Microtiter Plate Assay according to the manufacturer's instructions with a bovine serum albumin standard (BioRad).

2.12. Reverse transcription-polymerase chain reaction (RT-PCR) sequencing

One half-ml of vaccine on aluminum hydroxide (lot 0574) was centrifuged in a 1.5 ml eppendorf tube at 14 K rpm, 20 min, 4 °C. The supernatant was carefully removed and 25 µl of desorption buffer (mixture of 4 ml 1 M Na₂HPO₄, 200 µl of 5.6% EDTA, pH 8.0, 1 ml 1% tween-20, 1 ml 1% gelatin, 3.8 ml H₂O, final pH 8.5) was added to the pellet. The tube was vortexed for 30 s then incubated at 37 °C for 2 h with frequent agitation. The sample was centrifuged at 14 K rpm, 20 min, 4 °C. The supernatant was transferred to a fresh tube and the pellet was extracted as before two more times. The supernatant fluids from the three extractions were combined and viral RNA was prepared using a commercial RNA isolation kit (Stratagene). For reverse transcription (RT), the viral RNA was dissolved in 10 µl of diethylpyrocabonate (DEPC)-treated water. RT was carried out with cloned M-MLV reverse transcriptase (Superscript II, Life Technologies), 3 µl RNA, 1 mM dNTPs, and a primer (A5485) corresponding to nucleotide 5485–5465 (antisense strand) of JE SA14-142 [28]. RT reactions were carried out at 50 °C for 1 h. The DNA product from RT, without prior digestion with RNase H, was used as template for amplification by polymerase chain reaction (PCR). For PCR, the DNA was heated at 95 °C for 1 min then amplified using the Expand High Fidelity PCR System (Boehringer-Mannheim) with the primer pairs S5050 (nuc 5050–5070, sense strand) and A5431 (nuc 5431–5411, antisense strand). The PCR conditions were: 94 °C for 2 min; 60 °C for 30 s, and 68 °C for 1 min (one cycle), then, 94 °C for 15 s; 60 °C for 30 s, and

68 °C for 2 min (29 cycles), finally, an extension at 68 °C for 10 min. The PCR product was purified using a commercial chromatographic purification kit (Qiagen, inc.). Sequencing reactions were carried out in both the forward and reverse directions using primer S5071 (nuc 5071–5087) and primer A5414 (nuc 5414–5388) on an automated ABI PRISM Sequencer, Model 377 (ABI). Sequence data was compared with the published sequence of JE SA₁₄-14-2 [28].

2.13. Mouse immunogenicity assays

Immunogenicity assays were performed in 4–5 week old, outbred, female, Swiss-ICR mice. Animals were inoculated with graded doses of JE-PIV, lot 0574, or with commercially obtained licensed vaccine, Biken JE-Vax. Injections of 0.1 ml were administered with a 26-gauge needle and 1 cc syringe in the hindquarters region by the subcutaneous (sc) route. Inoculations were given on days 0 and 28 and animals were bled by retro-orbital puncture using sterile glass capillary pipettes on day 42. Sera from individual animals were tested for anti-JEV antibody in the virus plaque-reduction neutralization assay.

2.14. Mouse protection assays

Protection assays were done in 3-week-old, outbred, female, Swiss-ICR mice. Animals were inoculated with graded doses of JE-PIV, lot 0475, adjuvanted with 0.1% aluminum hydroxide, or with commercially obtained licensed vaccine, Biken JE-Vax. Injections of 0.2 ml were administered with a 26-gauge needle and one cc syringe in the hindquarters region by the sc route. Inoculations were given on days 0 and 14 and the mice were challenged with JEV (SA₁₄ strain) parent virus on day 28. Animals were bled immediately before challenge by retro-orbital puncture using sterile glass capillary pipettes. The challenge virus was administered by intraperitoneal (ip) injection using a 26-gauge needle and one cc syringe. Approximately 1000 median lethal doses (LD₅₀) of virus, (previously determined by titration), contained in 300 µl of EMEM was administered per animal. Concurrent with the ip virus challenge, the animals were injected intracranially with 30 µl of sterile saline delivered with a 27-gauge, 1/4-in. needle and 0.5-cc syringe to disrupt the blood-brain barrier. Animals were observed daily for signs of encephalitis. Animal survival was measured as a function of time for each vaccine dose tested.

2.15. Statistics

Probit analysis (least squares linear regression) implemented on a microcomputer running the SPSS program (SPSS, Chicago, IL) was used to calculate virus neutral-

izing antibody titers and to perform survival analysis after virus challenge. This program generates the appropriate 'P' values and 95% confidence intervals (CI) that were used to determine the statistical significance of the mouse protection assay results.

3. Results

3.1. Adaptation of JE virus to Vero cells

JE SA₁₄-14-2, PDK-8, virus was adapted to replicate in Vero cells by serial, low multiplicity passage. After two passages, there was an increase in virus titer of ~1000-fold, from 4 log₁₀ pfu/ml to greater than 7 log₁₀ pfu/ml, indicating adaptation. No further increase in virus yield was observed upon further passage (data not shown). A GMP vaccine master seed was made at Vero passage level-4 and a production seed was made at passage level-5. The production seed virus was used to infect Vero cells for making the vaccine.

3.2. cGMP Production of vaccine

Vaccine virus was harvested from infected Vero cell culture supernatant fluids on days 3, 5, 7 and 9 post-infection. As shown in Table 1, the virus titer from the harvests was 7–8 log₁₀ pfu/ml, with the maximum titer on day 7 post-infection. The supernatant fluids were pooled and concentrated, and the virus was purified on sucrose gradients. A small aliquot of the purified virus pool was electrophoresed on a SDS-polyacrylamide gel and Western blotted [26] with JE polyclonal antibody to visualize antigenic bands and stained with Coomassie blue to visualize total proteins. Only one major band with an apparent molecular weight (M_W) of 53 kilo-Daltons (kDa), consistent with that of E protein [29], was observed on the Western blot and the Coomassie-stained gel (data not shown).

The virus pool was diluted and filtered, after which the recovery of plaque-forming units was ~20% (Table 1). The virus was inactivated with 0.05% formalin at a constant temperature of 22 °C, conditions established by previous experiments (data not shown). Samples were removed daily from the vaccine, neutralized with sodium bisulfite, and tested for residual live virus by inoculation of Vero cells (Table 2). A total of 48 h after addition of formalin, the vaccine was re-filtered and transferred to a fresh flask. No infectious virus was recovered after 72 h and the incubation was continued for an additional 168 h to ensure complete inactivation. The residual formalin was then neutralized with sodium bisulfite, and the vaccine was dialyzed against several volumes of PBS. The bulk vaccine, designated as lot 0475, was tested for residual infectivity by inoculating 5% of the lot volume onto Vero cell cultures. Complete

inactivation of infectivity was demonstrated by the absence of virus plaques and cytopathic effect (Table 2). After the addition of 0.1% aluminum hydroxide and 0.01% thimerosol, the vaccine was bottled as JE-PIV lot 0574.

3.3. Immunogenicity of the vaccine in mice

The ability of the JE-PIV to elicit virus-neutralizing antibodies in mice was determined. Groups of ten adult outbred mice were immunized with graded doses of

Table 1

In process yields during cGMP manufacture of a purified, inactivated JE vaccine

Sample	Volume (ml)	Pfu/ml	HAU ^a	Protein (ug/ml)	Percent yield ^b
1st Harvest	2420	3×10^7	16	27.9	—
2nd Harvest	2400	8×10^7	16	18.1	—
3rd Harvest	2400	1.3×10^8	16	14.0	—
4th Harvest	2430	5.5×10^7	16	ND	—
Virus concentrate	350	1.8×10^9	512	475.1	100
Post-protamine	364	1.9×10^9	2048	270.2	110
Sucrose gradient pool	65	2.3×10^9	2048	327.4	24
Diluted sucrose pool	500	2.6×10^8	64	31.81	21
Filtered sucrose pool	500	2.4×10^8	ND ^c	20.9	19

^a Virus hemagglutinating units.

^b Based on recovery of virus plaque forming units (pfu), with recovery of pfu in the virus concentrate set at 100%.

^c Not determined.

Table 2

Formalin inactivation kinetics of the purified vaccine virus

Sample	Time post-inactivation (h)	Plaques detected by Vero cell-amplified virus assay	Residual virus titer by direct assay on Vero cells, PFU/ml
1	0	Positive	2.4×10^8
2	24	Positive	2.8×10^3
3	36	Positive	2.8×10^2
4	48	Positive	5.8×10^1
5	60	Positive	5
6	72	Negative	None
7	84	Negative	None
8	108	Negative	None
9	132	Negative	None
10	156	Negative	None
11	180	Negative	None
12	240	Negative	None

Table 3

Virus neutralizing antibody titers (PRNT₅₀) in mice vaccinated with JE PIV lot 0574 or commercial JE Vax

Vaccine	Dose (ng)	PRNT ₅₀ GMT	PRNT ₅₀ Range	Seroconversion rate	Immunizing dose 50% (ID ₅₀)
JE PIV	800	3842	1280–7781	10/10	4 ng (2–7 ng, 95% CI)
	160	1373	168–3026	10/10	
	32	113	34–563	10/10	
	6	ND ^b	ND	7/10	
	1	ND	ND	1/10	
JE Vax ^a	1200	733	283–3754	10/10	15 ng (14–17, 95% CI)
	240	117	18–863	10/10	
	48	90	55–150	7/10	
	10	ND	ND	6/10	
	2	ND	ND	1/10	

^a For JE-Vax, the virus protein concentration as reconstituted from the vial was 6 micrograms (6000 ng) per ml from information supplied by the manufacturer.

^b Not determined.

Table 4

Protection of mice vaccinated with JE PIV vaccine bulk lot 0475 or commercial JE Vax

Vaccine	Dose (ng)	PRNT50 GMT	Seroconversion rate	Survival rate	Effective dose 50% (ED ₅₀) ^b
JE PIV ^a	1500	400	10/10	10/10	2.6 ng (0.2–7.6 ng, 95% CI)
	150	170	10/10	10/10	
	15	20	10/10	8/10	
	1.5	10	3/10	4/10	
JE Vax	600	560	10/10	10/10	1.5 ng (0.6–3.7 ng, 95% CI)
	60	360	10/10	10/10	
	6	20	9/10	9/10	
	0.6	10	4/10	2/10	

^a Aluminum hydroxide (0.01%) was added to JE PIV bulk lot 0475 prior to inoculation of mice.^b Calculated ED₅₀s were based on survival rates measured after virus challenge.

JE-PIV, lot 0574, or with licensed JE vaccine (JE-Vax). Animals were given two inoculations of vaccine 4 weeks apart and blood was obtained 2 weeks following the second inoculation. Sera were tested for JE virus-neutralizing antibodies (Table 3). The dose of vaccine required to elicit detectable titers (PRNT₅₀ > 1:10) of neutralizing antibody in 50% of vaccinated animals (i.e. the Immunizing Dose 50%, ID₅₀) was calculated by Probit. The ID₅₀ for JE-PIV was 4 ng (95% CI 2–7 ng), which was less than the ID₅₀ of 15 ng (95% CI 14–17 ng) for JE-Vax. The lowest dose of the JE-PIV tested that elicited neutralizing antibody in 100% of vaccinated mice was 32 ng.

Table 5

Final container specifications and results of GLP testing of JE PIV lot 0574

Test	Specification	Result
Color	White	Satisfactory
Appearance	Opaque	Satisfactory
Uniformity	Non-homogeneous ^a	Satisfactory
Sterility	No Contamination	Satisfactory
General safety	Satisfactory	Satisfactory
Mouse ED ₅₀	<5 ng (protection from challenge)	2.6 ng
Endotoxin	<5 EU /0.5 ml	0.12 EU/0.5 ml
Mercury content	For information only	26.4 ug/0.5 ml
Aluminum content	<0.3 mg/0.5 ml	0.29 mg/0.5 ml
Identity by RT-PCR sequencing ^b	>95% sequence homology	99.5%
Protein concentration	For information only	4 ug/ml
PH	7.2–7.6	7.5

^a Signifies a non-uniform suspension which became homogeneous when shaken^b Vaccine was identified as JE by homology with the nucleotide sequence of Japanese encephalitis virus strain SA14-14-2 (see Section 2).

3.4. Protection of vaccinated mice against lethal virus challenge

The protective efficacy of the JE-PIV was determined by live virus challenge. Groups of ten weanling, outbred mice were vaccinated with graded doses of JE-PIV, bulk lot 0475, adjuvanted with aluminum hydroxide, or with commercially obtained JE-Vax. The animals were boosted on day 14 with the same dose of each vaccine, and challenged on day 21 with ~1000 lethal 50% mouse doses (LD₅₀) of parental JE SA₁₄ virus (Table 4). The dose of each vaccine required to protect 50% of vaccinated animals (Effective Dose 50%, ED₅₀) was calculated by Probit. For JE-PIV it was 2.6 ng (95% CI 0.2–7.6 ng) and for JE-VAX it was 1.5 ng (95% CI 0.6–3.7 ng). The lowest dose of JE-PIV in this experiment that protected 100% of vaccinated animals from death was 150 ng. Both vaccines were less effective without a booster (data not shown). Although the levels of neutralizing antibody at the time of challenge increased with increasing doses of vaccine, no correlation between antibody levels and protection could be established from these experiments.

3.5. Final vaccine specifications and pre-clinical safety testing

Prior to clinical trials in volunteers, JE vaccine Lot 0574 was subjected to extensive GLP testing, the results of which are summarized in Table 5. The identity of the vaccine was established by RT-PCR sequencing and comparison with the published sequence of JE SA14-14-2 [28]. In the region from nucleotide 5089–5394 the vaccine differed from JE SA14-14-2 only at nucleotide position 5311, which was T in the vaccine and C in the published sequence. Therefore, by all criteria vaccine lot 0574 was acceptable for use in clinical trials.

4. Discussion

The goal of this work was to make a new (PIV) against Japanese encephalitis virus for human use, adhering to the guidelines set forth by current Good Manufacturing Practices (cGMP). Although the existing licensed JE vaccine, made from virus-infected suckling mouse brain, purified, and inactivated with formalin, has been demonstrated to be safe and effective at preventing encephalitis, it has come under increased scrutiny for its reactogenicity, its production in neural tissue, and its high cost per dose. A candidate second-generation PIV produced in certified Vero cells is described in the present report.

JE virus strain SA₁₄-14-2, passaged eight times in PDK cells, was used as the seed virus for producing the new JE-PIV. This virus was previously demonstrated to be attenuated and safe for humans [18]. For vaccine production, it was adapted to Vero cells, which were pre-tested and banked. These cells have several desirable features: (1) they can be maintained in serum-free medium while supporting the growth of JE viruses to high titer; (2) the cell culture supernatants can be harvested several times for making one, large production lot; and (3) the absence of serum products increases the potential safety of the vaccine and enables purification to be accomplished by simple, efficient and inexpensive methods. Together, these advantages would appear to outweigh the slightly higher overall yield of virus obtained from suckling mouse brain. It must be mentioned, however, that there are still some concerns about the use of Vero cells to make parenterally-administered vaccines, which can be summarized as follows: (1) Vero cells at high passage level have been linked to tumor formation in immuno-compromised experimental animals; (2) Vero cell DNA has the potential to transform cells in culture; (3) Vero cell proteins may also have the potential to induce cellular transformation; and (4) Vero cells may harbor undetected adventitious agents. These issues were given full consideration when developing the methods for producing the new candidate JE-PIV. Vero cells at low passage level, demonstrated to be non-tumorigenic in animals, were used for making the vaccine. Cellular DNA was removed from the vaccine by precipitation with protamine to levels below current USFDA guidelines. Although acceptable limits for Vero cell proteins have not yet been established, the sucrose gradient purification method employed resulted in removal of the majority of contaminating cellular proteins. The vaccine was also tested and found negative for reverse transcriptase activity using the most sensitive assay available, and testing in cell culture and animals failed to reveal any evidence of adventitious agents. These data indicate that a JE-PIV prepared in Vero cells has an acceptable level of safety.

The existing licensed JE vaccine, JE-Vax, has proven efficacy, demonstrated in a large-scale clinical field trial [11]. Field efficacy trials to compare candidate second-generation JE vaccines with JE-Vax will have to be extremely large, making it unlikely that new vaccines will undergo such extensive testing prior to licensure. It may be possible to use a vaccine's ability to elicit virus-neutralizing antibodies as a surrogate marker for protection [30]. In that case, second-generation JE vaccine candidates will have to demonstrate equivalent or greater immunogenic potency compared with the existing licensed vaccine. In the present work, the mouse model was used to evaluate the potency of the new JE-PIV compared with JE-Vax. The results showed that at equivalent doses both vaccines protected mice and JE-PIV was more immunogenic.

One potential advantage of the new JE-PIV is that it is alum adjuvanted but contains no additional additives or stabilizers which may contribute to unwanted reactogenicity. Because of this, it may be possible to formulate future lots of JE-PIV with newer, more potent adjuvants, thereby increasing the vaccine's cost-effectiveness. However, the use of such adjuvants with JE-Vax may be contraindicated, since they might potentiate allergic reactions against the gelatin stabilizer or the very small amount of myelin basic protein still present after purification.

In conclusion, the results presented here suggest that the processes and materials used for production of the new JE-PIV will result in a product that is safe, effective and economical. The manufacturing methods are sufficiently robust, demonstrating good scalability, efficiency, and product yield. The cGMP lot described here is currently undergoing phase I clinical trials in order to establish product safety. Additional safety and efficacy data will also be gathered from tests performed in non-human primates. The new JE-PIV should be suitable for use not only in developed countries but also in developing countries where it is most needed.

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