Japanese Encepharitis Live Vaccine Protocol

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Boran Pharmaceuticals
Seoul, Korea

CONFIDENTIAL

Proposed by:

BORAN PHARMACEUTICALS
3F, Koryo Academytel, 437-3
Ahyun-Dong, Mapo-Gu
Seoul 121-010
SOUTH KOREA

Communications of inquiries related to the information contained in this file should be directed either to:

Sunheang Shin
R&D Team Leader/Pharmacist
3F, Koryo Academytel, 437-3
Ahyun-Dong, Mapo-Gu,
Seoul 121-010, Korea
Tel: 82-2/365-7000
Fax: 82-2/365-7009
E-mail: sunheang@soback.kornet21.net

CONTENTS

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BORAN PHARMACEUTICALS
3F, KORYO ACADEMYTEL I
437-3, AHYUN-DONG, MAPO-GU
SEOUL 121-010, THE REPUBLIC OF KORI
TEL : (82-2) 365-7000
FAX : (82-2) 365-7009

Dr. Mathura Shrestha
Chairman
Nepal Health Research Council
P.O Box 7626
Ram Shah Path
Kathmandu, Nepal

Dear Dr. Mathura,

We are pleased to submit you the attached materials for your review and approval for the effectiveness study of the Japanese encephalitis vaccine (live attenuated) in Nepal. For your information, the SA14-14-2 vaccine has been licensed in China since 1988. We got the approval for the safety and effectiveness in Korea in 1997 and therefore there is already some regulatory background available.

If you had any question or comment on it, please contact me.

With best regards,

Sunheang Shin
R&D Team Leader
Boran Pharmaceuticals

Summary of Documents

- Background
- General Description of JE Vaccine (live, attenuated)
- Relationship of Chinese Vaccine to Boran Vaccine
- Virology
- Preclinical Studies
- Clinical Safety
- Clinical Efficacy & Effectiveness

Summary

Background

JE is a mosquito-borne flaviviral infection which is the leading recognized cause of childhood encephalitis in Asia. About 35,000 cases and 10,000 deaths are reported to occur annually in the region. By any standard, JE is a major public health problem for Asia that potentially can be controlled by effective vaccines.

After an infectious mosquito bite, viral replication occurs locally and in regional lymph nodes. Virions disseminate to secondary sites where further replication contributes to an augmented viremia. Invasion of the CNS probably occurs from the blood, by antipodal transport of virions through vascular endothelial cells. Infection in the CNS spreads by viral dissemination through the extracellular space or by direct intercellular spread. Sensitized T-helper cells stimulate an inflammatory response by recruiting macrophages and lymphocytes to the perivascular space and parenchyma where the inflammatory response clears infected neurons with the subsequent formation of glial nodules. Why only one in several hundred infections develops into symptomatic neuroinvasive disease is unclear. Factors that contribute to neuroinvasion include age and potentially genetic and acquired host factors. Circulating antibody plays a critical role in modulating infection by limiting viremia in the pre-neuroinvasive phase. Both JE virus-specific and heterologous (e.g. dengue) antibodies contribute to protection, and low levels of neutralizing antibody may be sufficient to prevent viremia.

Clinically, high CSF alpha-interferon levels and low CSF virus-specific IgM and IgG antibodies have been associated with a fatal outcome, suggesting that delayed or poor local antibody response and uninhibited CNS viral proliferation determines outcome. Conditions that compromise the integrity of the blood-brain barrier have been suspected to increase risk for neuroinvasion and neurodissemination. Several observations suggest that dual infection with another infectious agent, especially neurocysticercosis, is a risk factor. The most widely used diagnostic method is IgM-capture ELISA. Specific IgM can be detected in CSF, serum or in both in approximately 75% of patients within the first 4 days after illness onset and, nearly all patients are positive 7 days after the onset of clinical signs and symptoms.

The great majority of infections are inapparent and only one in 250 infection results in symptomatic illness. The principle clinical manifestation of illness is encephalitis and milder clinical presentation, such as aseptic meningitis and simple febrile illness with headache, usually escape recognition. The incubation period is 5 to 15 days. Illness usually begins with an abrupt onset of high fever, change in mental status, gastrointestinal symptoms and headache, followed gradually by disturbances in speech or gait or other motor dysfunction. Irritability, vomiting, and diarrhea or an acute convulsion may be the earliest signs of illness in an infant or child. Seizures occur in more than 75% of pediatric patients and less frequently in adults. Conversely, a presentation with headache and meningismus is more common in adults than in children.

Five to 30% of cases are fatal with some deaths occurring after a brief prodrome and fulminant course lasting a few days and others, after a more protracted course of persistent coma. Young children (under 10 years) are more likely to die, and if they survive, they are more likely to have residual neurological deficits. Overall, approximately one third of surviving patients exhibit serious residual neurological disability. No specific therapy is available, but supportive treatment can reduce morbidity and mortality significantly.

Several different JE vaccines are used principally in Asia to protect local populations. One of those, an inactivated mouse brain-derived JE vaccine, is marketed in developed countries, including the USA, for travelers to Asia and for expatriates and especially, military personnel. Sporadic cases have been reported in travelers from North America, Europe, Russia, Israel, Australia and paradoxically, in Japanese and Taiwanese tourists to other endemic areas of Asia. Although travelers who remain in rural areas for extended periods are at greatest risk, well-publicized cases have been reported in travelers with brief itineraries in resorts or urban locations.

National vaccination programs in Japan, Taiwan and in Korea, using an inactivated mouse-brain derived vaccine have controlled the disease to the point of elimination. But in other countries, the expense and complexity of producing that vaccine, and the need for numerous doses, have limited vaccine implementation. Typically, two to three doses are given in the primary immunization series, followed by biennial or even annual boosters through childhood to maintain immunity. In addition to the limitations posed by multiple doses, as the vaccine has been introduced elsewhere to protect travelers, a high rate of hypersensitivity events has been reported among vaccine recipients in North America, Europe and Australia.

One hundred million doses of the SA14-14-2 JE Vaccine (Live, attenuated) have been distributed in China. However, licensure in the USA would provide a less reactogenic alternative to the inactivated mouse brain-derived vaccine, which now is the only internationally distributed JE vaccine.

General Description of JE Vaccine (Live, attenuated)

Workers at the National Institute for Control of Pharmaceutical and Biological Products (NICPBP) in Beijing pursued the attenuation of JE virus in PHK cells and derived strain SA14-14-2 that proved to be safe and immunogenic in animals and humans. The vaccine's efficacy was demonstrated in field trials and was licensed in the People's Republic of China (PRC) in 1988. Currently, 30 million doses are distributed annually in 13 southwestern provinces and in other selected regions. Expanded production and distribution within China are planned for the near future. Several hundred ampoules of master seed virus, prepared in 1993 from the fifth passage level of SA14-14-2 virus, are maintained in lots at the NICPBP in Beijing. Lyophilized seed virus (PHK5) is provided to each of two production institutes, where it is passaged once in PHK for the production seed (PHK6). PHK cells are obtained from 10-14 day-old golden Syrian hamsters maintained in closed colonies at the Chengdu and Wuhan Production Institutes.

Virology

Japanese encephalitis virus is one of 70 viruses in the *Flavivirus* genus of the Flaviviridae Family. The complete genomic sequences of JE virus and several other flaviviruses have been determined, including yellow fever virus, the prototype virus in the family. Morphologically, flaviviruses are spherical, approximately 40-50 nm in diameter, with a lipid membrane enclosing an isometric 30 nm diameter nucleocapsid core comprised of a capsid (C) protein and a single-stranded messenger (positive) sense viral RNA.

The molecular phylogeny of JE viruses, based on the 240 base nucleotide sequence of viral prM, divides JE isolates into four distinct genotypes, with a maximum divergence of 21 % among the isolates. The largest genotype consists of viruses from Japan, Okinawa, China, Taiwan, Vietnam, the Philippines, Sri Lanka, India and Nepal. A second genotype comprises isolates from northern Thailand and Cambodia and a third, from southern Thailand, Malaysia, Sarawak, Australia, and Indonesia. Five Indonesian isolates, two from Java, two from Bali, and one from Flores, similar to each other and distinct from other Indonesian isolates, form the fourth genotype. Co-circulation of multiple genotypes was observed only in Thailand and Indonesia. An antigenic analysis using five virus-specific monoclonal antibodies classified strains into four antigenic types, without correspondence to the genotypes above.

JE virus isolates from the same region but from different years show a high degree of nucleotide similarity. Sixteen Vietnam and 23 Okinawa strains of JE virus isolated between 1964-1988 and 1968-1992 differed by only 3.2% and 4%, respectively. However, viruses from the same region were distinguishable chronologically, before and after 1986 in Okinawa and before and after 1975 in Vietnam.

Pre-clinical Studies

The vaccine parent strain, SA14, was isolated in 1954 from Culex Pipiens larvae collected in Xian. After its isolation and 11 serial passages in weanling mice, the virus was attenuated through 100 passages in PHK cells at 36-37℃. Neurovirulence in monkeys had been lost at this passage level. Further plaque selection and cloning in chick embryo cells and subpassages in mice and hamsters by peripheral and oral infection were necessary, however, to obtain a stable aneurovirulent virus. The resulting SA14-5-3 strain no longer reverted to an established criterion of neurovirulence after intracerebral passage in suckling mice while remaining potent in mouse immunization-challenge studies. SA14-5-3 virus did not kill 3-week-old mice by either subcutaneous or direct intracerebral inoculation. Direct intrathalamic and intraspinal inoculation of the virus in monkeys resulted in no mortality or morbidity and a minimal degree of CNS inflammation, limited to areas around the injection sites. Histopathological changes were characterized by perivascular lymphocytic cuffs and focal mononuclear cell infiltration with rare direct neuronal degeneration or necrosis.

SA14-5-3 vaccine was shown to be safe in humans and field trials in endemic areas disclosed seroconversion rates greater than 85%. However, the vaccine's poor immunogenicity in flavivirus naive subjects from nonendemic areas suggested that SA14-5-3 virus, like previous live JE virus candidate vaccines, had been over-attenuated and did not replicate uniformly in humans. To increase immunogenicity, SA14-5-3 virus was serially passaged five times by subcutaneous inoculation of suckling mice, using skin, subcutaneous tissue and local peripheral lymph nodes as the passage material. After plaque selection and cloning twice in PHK cells, the SA14-14-2 strain was obtained. SA14-14-2 virus was equally attenuated but more immunogenic in mice, pigs and humans, producing seroconversion rates greater than 90% in nonimmune subjects.

The reduced neurovirulence of the SA14-14-2 strain was confirmed in three week old mice and monkeys. Compared with the parent SA14 strain, which killed weanling mice by subcutaneous or intracerebral inoculation with LD_{50}s in the range of $10^{5.5}$ to $10^{8.3}$ per ml, respectively, SA14-14-2 virus produced no mortality and only minor clinical signs in a few intracerebrally inoculated animals. Combined intrathalamic and intraspinal inoculation of rhesus monkeys produced no clinical illness and only minor inflammatory reactions in the substantia nigra and cervical spinal cord. Mice were more sensitive than monkeys to intracerebral infection, with some animals showing mild neuronal lesions in the cerebral cortex, hippocampus and/or basal ganglia. Compared with histopathological lesions produced by the parent SA14 virus, the inflammatory reaction to SA14-14-2 virus was greater and neuronal necrosis was significantly less. In five week old mice inoculated intracerebrally with the virus pair, ultrastructural studies showed the parent virus produced cytopathological changes in the majority of neurons, particularly in the rough endoplasmic reticulum and Golgi apparatus of the neuronal secretory system, while it could not be confirmed that the vaccine strain replicated at all and neurons appeared normal.

Further evidence of the strain's reduced neurotropism comes from experimental studies in athymic nude mice. No deaths or histopathological abnormalities were observed after intraperitoneal or subcutaneous inoculation of a viral dose greater than 10^7 TCID50 and virus could not be recovered from brain tissue. Although cyclophosphamide increases susceptibility of mice (and monkeys, as discussed earlier) to virulent JE virus, immunosuppresion with cyclophosphamide did not lead to encephalitis in mice inoculated peripherally with SA14-14-2 virus. The strain also did not kill intracerebrally inoculated weanling hamsters. Phenotypic characteristics of the vaccine strain (PHK8) such as small plaque size and reduced mouse neurovirulence were stable through at least 10 additional PHK cell culture passages.

Compared with two doses of inactivated P-3 vaccine, a single dose is more immunogenic and potent in protecting mice and guinea pigs against challenge, as measured by survival after intracerebral inoculation or suppression of viremia respectively. Six months after immunization, when neutralizing antibody titers declined to undetectable levels (<1:5), mice receiving attenuated vaccine were protected at higher rates (88%) than mice receiving inactivated vaccine (33%). Adoptive immunity, by transfer of immune spleen cells from immunized mice (50% protection versus 10%), and passive protection from immune serum (80% versus 33%), were better in mice immunized with live vaccine. Induction of cellular immunity also was shown by higher levels of protection in cyclophosphamide suppressed immunized mice (see above). Attenuated vaccine provided more effective protection than inactivated P-3 vaccine against a spectrum of JE strains isolated in China.

The underlying molecular basis of its neuroattenuation still is under active investigation. The nucleotide sequence of the neurovirulent parent SA14 virus differs from SA14-14-2 and two other attenuated SA14-2-derived vaccine viruses in only seven amino acid substitutions found in all three attenuated strains. Four were in the envelope protein (E-138, E-176, E-315 and E-439), one in nonstructural protein 2B (NS2B-63), one in NS3 (NS3-105), and one in NS4B (NS4B-106). Studies of other attenuated JE viral strains have shown the spectrum of mutations associated with phenotypic attenuation. ML-17, a pig vaccine strain derived by serial passage in primary monkey kidney cells, contains six amino acid changes in the protein coding region and one nucleotide change within the 3' noncoding region (nt-10512). (Chang unpublished results) An amino acid change at E-138, also present in SA14-14-2 virus, was shown to be sufficient for mouse neuroattenuation when introduced into a JE cDNA infectious clone. The other five changes are unique in ML-17 virus, E-146, NS3-192, NS4a-72, and NS4B-274 and -315. Only six passages of virulent Nakayama and 826309 viruses in HeLa cells (HeLa p6) resulted in significantly reduced neuroinvasiveness and nerovirulence for mice and altered receptor-binding activity. Nucleotide sequences of their structural protein genes revealed that the viruses differed by eight and nine amino acid mutations, respectively. Attenuated viruses also have been obtained by selecting neutralizing-resistant variants. Attenuation was associated with single base changes resulting in single E protein amino acid changes and was linked with altered early virus-cell interactions but not with replication.

Clinical Studies

Safety

An estimated 100 million children have been immunized with the SA14-14-2 live-attenuated vaccine without apparent complication. Clinical monitoring of experimentally immunized subjects has documented the absence of local or systemic symptoms after immunization, specifically, headache and symptoms that might be associated with neuroinvasive infection, and fever and signs and symptoms of systemic infection have not been observed after immunization. In a study of 867 children in which fever was monitored over a 21 -day period after immunization, temperatures above 37.6° C were recorded in fewer than 0.5% of vaccinees and fever-onset days were distributed throughout the observation interval, mitigating against a vaccine-related febrile illness after a specific incubation period. In the same study, symptoms were recorded from 588,512 other vaccines, fever was reported in 0.046% of subjects, rash in 0.01 %, dizziness in 0.0003%, and nausea in 0.0003%, however, these rates are difficult to interpret in the absence of similar observations in controls.

A block randomized cohort study of 13,266 vaccinated and 12,951 unvaccinated 1-2 year old children, followed prospectively for 30 days, was reassuring in confirming the vaccine's safety. No cases of encephalitis or meningitis were detected in either group and rates of hospitalization, new onset of seizures, fever lasting more than three days, and allergic, respiratory and gastrointestinal symptoms were similar in the two groups. The observations excluded a vaccination-related encephalitis risk above 1 in 3,400.

The rates of clinical encephalitis among children vaccinated in field trials provide additional reassurance that SA-14-14-2 virus does not itself cause encephalitis at a detectable rate. Rates of clinical encephalitis in children receiving SA-14-14-2 vaccine -- 1.16 to 6.75 per 100,000 -- are lower than reported population based incidence rates of childhood encephalitis (15-30 per100,000).

Efficacy and Effectiveness

Several small immunogenicity studies of the SA14-14-2 vaccine have been reported, with variable results. After a single dose, antibody responses are produced in 85 to 100% of nonimune l- to 12-year-old children, with a response gradient that parallels progressive vaccine dilution. Lower seroconversion rates were obtained with vaccine dilutions that had infectious titers less than $10^{6.7}$ $TCID_{50}$ per mL, which has been established as the minimal standard of vaccine infectivity.

Because of variable immune response rates after one dose, SA14-14-2 vaccine is given in a schedule of two doses separated by a year, according to the custom of annual spring campaigns. The immunogenicity of two doses given at intervals of either 1 or 2.5 months was shown in 12- to 15-year-old children. Response rates were similar: 75 to 100% after one dose and 94 to 100% after two doses (two vaccine lots were compared), but there was a trend toward better seroconversion with the longer interval, and GMTs were approximately two-fold higher (65-89 versus 115-158, respectively).

Efficacy trials in children 1 to 10 years old have consistently yielded high protection rates above 98%. In the 1991 Yunnan field study, neither of the two cases in vaccinated children produced serious illness, but three deaths occurred in the unvaccinated cohort, and more than 50% of the remaining cases were considered severe. In the Guizhou study, equally good protection was observed through a second year after a booster dose was given.

A study measuring the effectiveness of the SA14-14-2 vaccine, using case-control methods, disclosed protection levels similar to those estimated by previous efficacy studies. When immunization histories were compared among 56 hospitalized laboratory-confirmed JE cases and 1,299 age-matched village controls, the vaccine's effectiveness was 80% for one dose (95% CI = 44-93%) and 98% for two doses (95% CI = 86-99.6%). Because of uncertainties about the methodological approach of earlier efficacy studies, the consistency of this result with previous estimates was reassuring. Furthermore, effectiveness is a measure of the vaccine's performance under the usual circumstances of health care delivery rather than the artificial conditions of a study, which is additional evidence of the vaccine's robustness.

Scientific Rationale for Use of JE Vaccine (Live, attenuated)

Scientific Rationale for Use of JE Vaccine (Live, attenuated)

The development and use of JE vaccines in endemic areas of Asia have resulted in significant reductions in morbidity and mortality associated with JE infection. The live attenuated SA14-14-2 JE vaccine has been used in China to immunize more than 100 million persons. The safety and efficacy of that vaccine have been established in published clinical studies.

There are two major points that differentiate the SA14-14-2 vaccine from the currently licensed mouse brain-derived vaccine. First, the SA14-14-2 vaccine is a live attenuated product whereas the mouse brain-derived vaccine is an inactivated product. Second, the SA14-14-2 vaccine is cell culture-derived and does not contain contaminants associated with the brain-derived vaccine which are related to adverse events.

Overview of Japanese encephalitis as a disease and the medical need for vaccines

- Excerpts from chapter on JE vaccines by TF Tsai, GJ Chang, YX Yu

Chapter 24

JAPANESE ENCEPHALITIS VACCINES

Theodore F. Tsai, M.D., M.P.H.
Gwong-Jen J. Chang D.V.M., PhD.
Yong Xin Yu

BACKGROUND

Japanese encephalitis (JE), a mosquito-borne flaviviral infection, is the leading recognized cause of childhood encephalitis in Asia. Approximately 35,000 cases and 10,000 deaths are reported annually but in many locations the disease is not under systematic surveillance and official reports undoubtedly underestimate the true number of cases (Figure 24-1).[1-3] Although the disease is transmitted only in Asia, with more than 3 billion people and 60% of the world's population, regional JE-associated morbidity may exceed worldwide morbidity from herpes encephalitis, estimated at 5 per 1 million per year, or approximately 30,000 cases worldwide.[4,5] With the near eradication of poliomyelitis, JE now is the continent's leading cause of childhood viral neurological infection. By any standard, JE is a major public health problem that potentially can be controlled by proven effective vaccines.

Summer-fall encephalitis outbreaks consistent with JE were recorded in Japan as early as 1871, of which the largest, in 1924, led to more than 6000 cases, 60% of them fatal.[6] A filterable agent from human brain tissue was isolated in rabbits that year, and in 1934, Hayashi transmitted the disease experimentally to monkeys.[7] Soon after, the availability of JE and related St. Louis encephalitis viral isolates made possible the serological confirmation of encephalitis cases occurring elsewhere in the region, including a cluster of cases occurring in 1934-35 in Beijing.[8] The virus initially was called Japanese B encephalitis (the modifying "B" has since fallen into disuse) in deference to Von Economo's type A encephalitis, which had different clinical and epidemiological characteristics. The mosquito-borne mode of JE transmission was elucidated with the isolation of JE virus from *Culex tritaeniorhynchus* mosquitoes in 1938 and subsequent field studies established the role of aquatic birds and pigs in the viral enzootic cycle. Viruses isolated from human cases in Japan in 1935 and in Beijing in 1949 provided the prototype Nakayama, Beijing, and P3 strains, respectively, that are in principal use in vaccine production today.

During the first half of this century, JE was recognized principally in temperate areas of the continent in the form of perennial outbreaks in Japan, Korea, and China.[1] Annual outbreaks of several thousand cases recurred in Japan until as recently as 1966 with a public impact that must have been further magnified by their concentration during the summer season. In Korea, after 5616 cases and 2729 deaths were recorded in 1949, epidemics continued every two or three years, culminating in an unprecedented 6897 cases in 1958 (Figure 24-2).[9] However, China has accounted for the majority of cases in the region - between 1965-75, more than a million cases were reported, 175,000 in 1971 alone (Figure 24-3).[10] Public health efforts placing a great emphasis on vaccination produced a dramatic decline in cases, however, coverage remains low in many provinces and in recent years, incidence in the rural population has remained stable. In Japan, Korea and Taiwan, the introduction of national immunization programs after 1965 led to the near elimination of the disease; however, the absence of reported cases is disarming since enzootic transmission of the virus in its enzootic cycle

continues in these locations and periodically, outbreaks have occurred, as in 1982, when 1197 cases were reported in Korea[11] (Figure 24-4).

Although sporadic viral encephalitis cases had been noted in northern Thailand, JE was not a recognized public health problem in Southeast Asia until 1969 when an epidemic of 685 cases was reported from the Chiang Mai Valley in the north of the country.[12] Yearly outbreaks producing thousands of cases and hundreds of deaths followed in the northern region and JE became recognized as a leading cause of childhood mortality and disability (Figure 24-5)[13]. Subsequently, the first of several epidemics was recorded in an adjacent area of the Chiang Mai Valley in Burma in 1974.[14] In Vietnam, since notifications were re-instituted in 1979, several thousand JE cases have been reported annually and the disease has been recognized as a public health threat in the densely populated deltas of the Mekong and Red Rivers.[15] Incidence rates exceeding 20 per 100,000 are reported from areas of the northern delta near Hanoi. The disease probably occurs with equal frequency in Laos and Cambodia where clinically and epidemiologically compatible cases have been reported but medical and public health infrastructure are lacking to confirm the etiology. Recent studies in Penang, Malaysia and Bali, Indonesia indicate that 40-50% of hospitalized encephalitis cases respectively are caused by JE underscoring the inadequacy of public health surveillance, since few cases previously had been reported from these locations and even the occurrence of JE had been questioned.[16,17] The continued public health impact of JE in the region has led to efforts in Thailand and more recently in Vietnam to implement programs of childhood immunization and vaccine production.[15,18]

JE transmission was first recognized in Southwest Asia after an outbreak occurred in 1948 in Sri Lanka. Sporadic cases and later, epidemics were recognized on the Indian subcontinent around Vellor.[19,20] Outbreaks recurred exclusively in southern India until 1973, when JE epidemics were reported in the north for the first time in the Burdwan and Bankura districts of West Bengal and afterwards in Bihar and Uttar Pradesh. Apparently novel occurrences of JE subsequently were reported from various states and the disease now is recognized to be hyperendemic in northern India and southern Nepal, central India (Andrha Pradesh), and southern India (Goa, Karnataka, and Tamil Nadu) (Figure 24-6). JE recently has been shown to occur as far west as the Indus valley in Pakistan.[21] The apparent spread to or amplification of JE in new areas has been correlated with agricultural development and intensive rice cultivation supported by irrigation schemes.[22] In Sri Lanka and southern Nepal, hyperendemic transmission of malaria and JE were documented to have followed deforestation and development in the Mahaweli River valley and Terai, respectively.[23,24] The potential spread of JE is being watched closely on Irian Jaya, Indonesia, in the irrigated Thar desert of Rajasthan, and other places under development where conditions receptive to viral transmission and amplification recently have been created. Recent novel introductions leading to outbreaks on Saipan island and the Torres Strait islands between New Guinea and northern Australia illustrate the potential for JE virus to be transferred over significant distances, possibly by viremic birds.[25,26] While development has led to the near elimination of JE in economically advanced Asian countries (Japan, Korea, Taiwan and Singapore) development in its earlier stages, emphasizing agricultural productivity, seems to have increased JE transmission.

Clinical Illness

The great majority of infections are inapparent and only one in 250 infections results in symptomatic illness.[27,28] The principal clinical manifestation of illness is encephalitis and milder clinical presentations, such as aseptic meningitis and simple febrile illness with headache,

usually escape recognition.[29–38] The incubation period is 5 to 15 days. Illness usually begins with an abrupt onset of high fever, change in mental status, gastrointestinal symptoms and headache, followed gradually by disturbances in speech or gait or other motor dysfunction. Irritability, vomiting, and diarrhea or an acute convulsion may be the earliest signs of illness in an infant or child. Seizures occur in more than 75% of pediatric patients and less frequently in adults. Conversely, a presentation with headache and meningismus is more common in adults than in children.

A progressive decline in alertness eventually leads to stupor and coma. A substantial proportion of patients become totally unresponsive and require ventilatory assistance. Generalized weakness and changes in tone, especially hypertonia and hyperreflexia, are common but focal motor deficits—including paresis and hemiplegia and tetraplegia, cranial nerve palsies (especially central facial palsy), and abnormal reflexes—also may be present. Sensory disturbances are seen less frequently. Central hyperpnea, hypertension, pulmonary edema, and urinary retention also may complicate the illness. Although symptoms suggest elevated intracranial pressure in many cases, papilledema and other signs of raised intracranial pressure rarely are seen, and in a controlled trial, dexamethasone therapy did not improve outcome.[39, 40] Signs of extrapyramidal involvement, including tremor, mask-like facies, rigidity, and choreoathetoid movements, are characteristic of JE but these signs may be obscured initially by generalized weakness.

Clinical laboratory examination discloses a moderate peripheral leukocytosis with neutrophilia and mild anemia. Hyponatremia reflecting inappropriate ADH secretion is a frequent complication. The CSF usually is under normal pressure. Pleocytosis ranges from a few to several hundred cells per cubic mm with a lymphocytic predominance; neutrophils may prevail in early samples. Cerebrospinal fluid protein is moderately elevated in about 50% of cases. Reduced CSF monoamine levels (homovanillic and 5-hydroxyindoleacetic acids) have been found in the acute phase of illness and in recovery but these reductions have not correlated consistently with clinical parkinsonism.[41]

Computed tomographic (CT) scans and magnetic resonance imaging (MRI) reveal low-density areas and abnormal signal intensities respectively in the thalamus, basal ganglia, pons and putamen.[42-46] Acute changes in the thalamus may be a helpful differentiating feature: in JE cases, T2 weighted MRI images more frequently disclose bilateral thalamic high intensity lesions representing hemorrhages and single photon emission CT, increased activity in the thalami and putamina, in comparison to encephalitis cases due to other causes.[45] MRI abnormalities may be seen in the spinal cord, underscoring that JE is an encephalomyelitis. Electromyographic changes reflecting anterior horn cell degeneration are detected, especially in patients with clinical wasting;however, abnormalities in somatosensory evoked potentials are rare, which is consistent with the infrequency of clinical sensory deficits. Delays in central motor conductance time reflect widespread involvement of white matter, thalamus, brainstem and spinal cord.[46] Electroencephalographic tracings typically show diffuse delta wave activity but alpha coma also may be seen. Imaging and neurophysiological abnormalities indicative of thalamic damage correlate with several of the clinical manifestations typifying the acute phase of illness.

Five to 30% of cases are fatal with some deaths occurring after a brief prodrome and fulminant course lasting a few days and others, after a more protracted course of persistent coma. Young children (under 10 years) are more likely to die, and if they survive, they are more likely to have residual neurological deficits. Overall, approximately one third of surviving patients exhibit serious residual neurological disability.[30,47–52] Principal sequelae include memory loss, impaired cognition, behavioral disturbances, convulsions, motor weakness or paralysis, and

abnormalities of tone and coordination. In children, motor abnormalities frequently improve or eventually resolve but behavioral changes and psychological deficits have been detected two to five years after recovery in up to 75% of pediatric cases; EEG abnormalities also may persist in the absence of detectable clinical signs.[53] Evidence of previous dengue immunity is associated with better outcome.

Poor prognosis has been associated with a short prodromal interval, clinical presentation in deep obtundation, respiratory dysfunction, prolonged fever, focal presentation, status epilepticus, and the presence of extrapyramidal signs or pathological reflexes[47, 54–58] In some locations, concurrent neurocysticercosis has been reported in over one third of JE cases with evidence of increased mortality in coinfected patients (see below).[59]

Anecdotal observations suggest that infection may fail to clear in certain individuals, with the possibility of clinical relapse several months after resolution of the acute illness.[60] In several cases, symptoms recurred and virus was recovered from persistently infected peripheral lymphocytes despite circulating antibody. Other recovered patients who were studied months after recovery had apparently asymptomatic viremias. The possibility of subacute or persisting infection in the CNS was demonstrated in 5% of patients whose CSF contained virus or viral antigen for three weeks or who had intrathecal IgM antibodies 50-180 days after onset.[61] The clinical significance of these observations and conditions under which JE virus persists in humans are unclear.

No specific therapy is available, but supportive treatment can reduce morbidity and mortality significantly. Mannitol and other modalities to control intracerebral pressure often are needed. Trihexphenidyl hydorchloride and central dopamine agonists have been used to treat acute extrapyramidal symptoms.[62] Neutralizing murine monoclonal antibodies developed in China have been reported to improve clinical outcome in small controlled clinical trial and licensure in that country has been sought.[63] Experimental studies in mice and monkeys also have suggested a potential benefit of interferon and, in an uncontrolled series of 14 patients treated with recombinant α-interferon, 13 survived; however, further studies have not been undertaken.[64,65] A number of antiviral compounds, including ribavirin, exhibit activity in vitro but have not been evaluated clinically.

Pathological abnormalities are found chiefly in the CNS; however, inflammatory changes in the myocardium and lung and hyperplasia of reticuloendothelial cells in the spleen, liver and lymph nodes have been described.[66] Cerebral edema and congested leptomeninges are visible on gross examination of the brain, and punched-out necrolytic lesions in the gray matter may be conspicuous.[66-72] Histopathological examination discloses a pattern of diffuse microglial proliferation with nodular formation around dead or degenerating neurons, in which viral antigen can be demonstrated by immunohistochemical staining.[40,68,72] Viral antigen is distributed principally in the thalamus, midbrain, hippocampus and temporal cortex but also in Purkinje and granular cells of the cerebellum and in the brainstem reticular formation. However, viral antigen also has been demonstrated in well preserved neurons independent of glial reaction, in some cases, well after the acute phase of illness, suggesting intracellular viral persistence. Gliomesenchymal nodules are seen in a parallel distribution within the brain and anterior horn of the spinal cord. In patients dying with residual neurological impairment several years after resolution of the acute illness, scarred rarified foci are found in a characteristic distribution in the thalamus, substantia nigra, and hippocampus.[73]

Protective Effects of Immunization

Inactivated Mouse Brain-Derived JE Vaccine

A neutralizing antibody titer of more than 1:10 generally is accepted as evidence of protection and post-vaccination seroconversion. Passively immunized mice that acquire this level of neutralizing antibody are protected against challenge from 10^5 LD50 of JE virus, a typical dose transmitted by an infectious mosquito bite. Indirect observations from human trials have associated efficacy with this criterion. Although individual laboratories employ test procedures of varying sensitivity to measure neutralizing antibody, results are surprisingly robust. Plaque reduction neutralization tests are used most frequently and procedural. differences such as choice of challenge virus strain, cell systems, addition of erogenous complement and choice of endpoints, ranging from 50 to 90% plaque reduction in serum dilution tests, affect test sensitivity. Some laboratories still employ log neutralization indices (LNI) in tests using a single serum dilution. However, despite procedural differences, neutralizing antibody titers in three laboratories (CDC, Japan NIH, Yale Arbovirus Unit) were shown to be highly correlated (unpublished observations, R DeFraites). No international standard of protective antibody units currently is established.

Immunogenicity. Among Asian children immunized with two doses of Nakayama or

Beijing-1 strain-derived vaccines, neutralizing antibody responses to the respective homologous vaccine strains are in the range of 94-100% and are lower to strains representing a heterologous antigenic group (selected recent studies shown in Table 24–10).[257-259] The proportion of vaccinees retaining detectable neutralizing antibodies and their geometric mean titers (GMT) declined rapidly in the year after the primary two dose series, so that only 78-89% of Nakayama vaccine recipients and 88-100% of Beijing-1 vaccine recipients still had protective levels before the scheduled one year booster. Antibody persistence was greater among Beijing-1 vaccine recipients. After booster immunization (third vaccine dose), antibody response rates were uniformly high, 100%.

Immunogenicity studies in Asian subjects should be interpreted in light of the immunological background of vaccinees. Although some studies have been carried out in nonendemic areas and in subjects without JE viral antibodies, in others, undetected exposures to JE, dengue, and other flaviviruses prevalent in Asia may have resulted in an augmented antibody response after immunization and apparently better immune responses. Where the influence of previous flaviviral infections was unlikely, vaccinees receiving two doses produced lower seroconversion rates and lower GMTs (Table 24–11 and Fig. 24–17A).[20,221-223] Moreover, as rapidly as 6 to 12 months after primary immunization with two doses, neutralizing antibody titers declined below 1:8 in 90% of vaccinees (Fig. 24–17B).[181] A 3-dose primary schedule was more immunogenic, resulting in seroconversion rates exceeding 90% and significantly higher neutralizing antibody titers.[20, 221-223] A comparison of long (days 0, 7, and 30) and short (days 0, 7, and 14) three-dose schedules disclosed uniform seroconversion in all subjects but significantly higher neutralizing antibody titers in vaccinees immunized over 30 days.

Vaccine prepared from the Beijing-1 strain appears to be more immunogenic, despite its smaller delivered volume, yielding higher seroconversion rates and higher antibody titers to heterologous Nakayama virus (Table 24-10).[260-262] Similar, but more marked differences were seen in comparative neutralization of field viral strains from Taiwan, paralleling those in experimentally immunized mice (Figure 24-16).[263] The clinical importance of these differences in strain reactivity is uncertain. Results of the efficacy trial comparing a monovalent Nakayama strain vaccine with a bivalent vaccine also containing Beijing-1 antigen showed the two were equally efficacious (see below).[264] JE vaccines produced locally in Thailand, India, Vietnam and Taiwan all employ the Nakayama strain and no field observations suggest a geographic pattern of vaccine failure. Neutralizing activity may be present below the threshold of detection in *in vitro* assays and T cell memory may have been established in vaccinees who appear to be seronegative, providing sufficient help to clear infections upon reexposure.

Although previous exposures to dengue and certain other flaviviruses probably enhance the immune response to JE vaccine, antibody responses did not differ in persons with a history of yellow fever (YF) vaccination, unlike the accelerated response to inactivated tick-borne encephalitis vaccine seen among YF vaccinated individuals.[265]

Vaccinees are exposed only to viral structural proteins and in contrast to recovered patients, they do not produce radioprecipitating antibodies to viral nonstructural proteins. Their T memory cell proliferative responses to a viral-like particle containing only structural proteins also differ from recovered patients, whose $CD4^+$ and $CD8^+$ cell responses also include viral nonstructural proteins.[131] The implications of these immune response differences are uncertain.

Impaired responses to vaccination were observed in infants with vertically acquired HIV infection compared to control seroreverting infants born to HIV infected women: five of 14 (36%) HIV-infected children and 18 of 27 (67%) control children developed JE antibodies after immunization (OR 0.3, p=0.06); among those with positive titers, the geometric mean titer (GMT) of HIV-infected children also was lower (15.1 vs. 23.8; p=0.17).[266] The response to additional

d the primary two doses was not studied. Immune response in other
promised states has not been studied systematically.[267]
icy. Efficacy of the Nakayama vaccine has been evaluated in two masked
placebo (tetanus toxoid) controlled field trials. In the first evaluation, a prototype of
accine was field-tested in 1965 in Taiwan; two doses yielded an 80% efficacy in the
immunization (Table 24–12).[268-270] A subsequent masked randomized placebo
d trial in Thailand compared the efficacies of the currently produced monovalent
ccine with a specially formulated bivalent vaccine also containing Beijing-1 antigen
–12).[264] Two doses of vaccine or placebo were given 1 week apart to children 1
id older. After a 2-year observation period, efficacies of the monovalent and
nes were identical with an overall efficacy of 91%. Lower risks of dengue and
rrhagic fever also were observed in the JE vaccinated groups; however, the
ere not significant. Experimental studies in monkeys suggest that immunization
ght provide cross-protection against West Nile virus.[125]
tence of Immunity and Protection. Studies in Asia to determine the persistence
ived immunity are complicated by natural infections with dengue, West Nile virus,
ruses and reexposure to JE virus itself, which act to reinforce and broaden
d immunity to JE virus.[101,102,122-124,153] Even with the potential for these reinforcing
eral studies in Asian and in Western subjects (see previous discussion) indicate a
cline in antibody levels in the first year after primary immunization with two doses
able 24-10).[206] Cross sectional serosurveys in Japan and Taiwan (see above)
indicate a rapid decline of immunity in childhood. Observations of vaccine efficacy
ield trial parallel these results; in the second year after immunization, protective
ed from 80 to 55% (95% confidence interval, 39 to 75%).[269] In the Thailand field
ent vaccine formulation, efficacy was shown through 2 years of observation.
up data are not available.
ind other data (see Table 24–11) indicate the need for boosters after a two-dose
zation series. A third dose generally has been given at 1 year and subsequently
-3 years (see Fig. 24–18). Booster doses are followed by significant rises in
ibody titer and uniform anamnestic responses in subjects who had reverted to
A small study of vaccinees receiving an Indian manufactured JE vaccine found
) retained neutralizing antibodies three years after a primary series of three
34 (91%) retained antibodies at 4.5 years, with respective GMTs of 71 and 32
owever, the boosting effect of naturally acquired flaviviral infections in these
be ruled out.[271]
l naive U.S. Army soldiers who received a three-dose primary immunization series
ive neutralizing antibody titers for at least 1 year (GMT, 76). Antibody titers at 12
changed from those observed 3 months after immunization (GMT, 78). A booster
2 months was followed by a significant anamnestic response (GMT, 1117).[225] In a
of subjects studied three years after the primary series, 16/17(94%) who had
to Asia or received a booster retained neutralizing antibody titers >1:10 and their
ears and at six months after primary immunization were unchanged.[221] Although
ns suggest that the first booster immunization is needed no sooner than two-
primary immunization, the interval for subsequent boosters has not been

nary Hamster Kidney Cell–Derived Japanese Encephalitis Vaccine

Two doses of inactivated PHK cell–derived vaccine, given 1 week apart, produced an LNI
ɜr than 50 in only 60 to 68% of children who had no prevaccination JE viral antibodies
ɜ 24-13).[272,273] Immunity wanes rapidly after primary immunization with two doses, and only
ɔf vaccinees have an LNI greater than 50 one year later. The rapid decline in antibody
es some justification for the vaccine's administration in spring campaigns before the onset
transmission season. A booster dose results in an anamnestic response in 93 to 100% of
ɜnts. After 3-4 years, seropositivity is maintained at an LNI of greater than 50 in 64% of
ees, and a subsequent booster dose is followed by 100% seroconversion.[194,274] Extensive
nized field trials among 480,000 children have demonstrated vaccine efficacies in the
of 76 to 95% (Table 24–14).[194, 275, 275]

Although regional trials in Wuxi and Nanjing disclosed partial protection against acquiring
icacies of 85 to 87%), more detailed clinical studies showed that cases in vaccinated
n were milder than those in unvaccinated children. None of the six cases in immunized
n resulted in death or neurological sequelae, whereas three of the 25 cases in
unized children were fatal and three led to sequelae. These observations suggest a better
efficacy than the reported protective efficacy.[194] A case-control study measuring vaccine
'eness in Henan province, China found that full immunization (two primary doses and
boosters until age 10) was 78% effective (95% CI 16-94%) in preventing the disease and
'mmunization was 68% effective (95% CI -29-92%). The relative risk of acquiring JE was
unimmunized children and 3.12 in partially immunized children.[276]

The aggregated data indicate that the inactivated P-3 vaccine has some utility in
ɜing the disease; however, the need for repeated booster doses and a relatively low
' are limiting.

Immune responses to single doses of concentrated or purified inactivated PHK cell
s (see previous comments) were similar to those observed after two doses of the
d vaccine. All subjects seroconverted, and respective geometric mean neutralizing
y titers were 45, 72, and 46.[194] No data on efficacy or persistence of immunity are
e and further work has been discontinued.

tenuated Japanese Encephalitis Vaccine

A comparison of SA14-5-3 and SA14-14-2 derived vaccines showed that the former was
ɪunogenic, producing seroconversion in only 61% of 13 vaccinees and a GMT of 5,
ɜd with a 92.3% seroconversion rate in subjects receiving a similar infectious dose of
ɬ-2 vaccine.[237] Several small immunogenicity studies of the SA14-14-2 vaccine have
ɔorted with variable results. After a single dose, antibody responses are produced in 85 to
nonimmune 1- to 12-year-old children, with a response gradient following progressive
dilution (Table 24–15).[237,238,277] Lower seroconversion rates were obtained with vaccine
that had infectious titers less than $10^{6.7}$ $TCID_{50}$ per ml, which has been established as
ɪal standard of vaccine infectivity.

ɜecause of variable immune response rates after one dose, SA14-14-2 vaccine is given
ɜdule of two doses separated by a year, according to the custom of administering JE
in annual spring campaigns. The immunogenicity of two doses given at intervals of
e or 2.5 months was shown in 12-15 year old children. Response rates were similar, 75-
ɜer one dose and 94-100% after two doses (two vaccine lots were compared) but there
ɜnd towards better seroconversion with the longer interval and GMTs were approximately
ɜigher (65-89 versus 115-158 respectively). If these results can be confirmed in infants,
ɬ-14-2 could be integrated into a routine childhood immunization schedule, potentially

improving vaccine coverage.[256] The effect of maternal immunity on antibody response in infants has not been examined.[278]

Efficacy trials in children 1 to 10 years old consistently have yielded high protection rates above 98% (Table 24–15).[246, 279,280] In the 1991 Yunnan field study, neither of the two cases in vaccinated children were seriously ill, but three deaths occurred in the unvaccinated cohort and more than 50% of the remaining cases were considered severe. In the Guizhou study, equally good protection was observed through a second year after a booster dose was given. Efficacy was shown with the more attenuated prototype SA14-5-3 vaccine, although protection was lower than that achieved with SA14-14-2 vaccine (see Table 24–8).[246, 279, 280]

A study measuring effectiveness of the SA14-14-2 vaccine, using case-control methods, disclosed protection levels similar to those estimated by previous efficacy studies. When immunization histories were compared among 56 hospitalized laboratory confirmed JE cases and 1299 age-matched village controls, the vaccine's effectiveness was 80% for one dose (95% CI, 44-93%) and 98% for two doses (95% CI, 86-99.6%).[281] Because of uncertainties about the methodological approach of earlier efficacy studies, the consistency of this result with previous estimates was reassuring. Furthermore, effectiveness is a measure of the vaccine's performance under the usual circumstances of health care delivery rather than the artificial conditions of a study which is additional evidence of the vaccine's robustness.

Interestingly, the vaccine's variable immunogenicity, the relatively low neutralizing antibody titers elicited and the need for two doses suggest that the strain may be over-attenuated. However, vaccination evidently provides sufficient immunological memory, supplemented possibly by natural exposures to the virus, to be highly effective in protecting against clinical illness.

Side Effects of Immunization

Inactivated Mouse Brain–Derived JE Vaccine

Local and nonspecific adverse events Local tenderness, redness, and/or swelling at the injection site occur in approximately 20% of individuals immunized with inactivated mouse brain–derived vaccines. Mild systemic symptoms, chiefly headache, low-grade fever, myalgias, malaise, and gastrointestinal symptoms, are reported by 10 to 30% of vaccinees (Table 24–17).[181, 222, 223, 257]

Neurological adverse events The vaccine's neural tissue substrate has raised concern about the possibility of postvaccination neurological side effects.[282] The manufacturing process purifies the infected mouse brain suspension extensively and myelin basic protein (MBP) content is controlled below 2 ng per ml, well below the dose considered to have an encephalitogenic effect in a guinea pig test system. However, measurements of other ADE-associated neural proteins in the vaccine (e.g. PLP, MOG, MAG and S100) have not been reported. Experimental immunization of guinea pigs and *Cynomolgus* monkeys with adjuvant and 50 times the normal dose of vaccine did not result in clinical or histopathological evidence of encephalomyelitis.[283, 284]

In 1945, in one of the first mass uses of mouse brain–derived JE vaccine, 53,000 American soldiers on Okinawa were immunized with a crude inactivated mouse brain suspension after a JE outbreak occurred on the island.[200] Acute vaccine-associated side effects, including the occurrence of acute neurological events, were monitored. Eight neurological reactions, principally polyneuritis, were observed. However, similar cases were reported concurrently in nonvaccinated individuals, and it is unclear whether the illnesses were vaccine-related. One case of Guillain-Barré syndrome, temporally related to JE immunization, was reported among

approximately 20,000 American soldiers immunized with the vaccine prior to U.S. licensure.

An early prospective study in Japan to detect vaccine-associated adverse events found no neurological complications occurring within a month after vaccination in 38,384 subjects receiving crude or purified vaccine.[282] A country-wide study to detect neurological complications found 26 emporally related cases (meningitis, convulsions, demyelinating disease, polyneuritis) between 957-1966, but rates and comparisons with unimmunized controls were not available. Passive urveillance of vaccine-related adverse events (AE) in Japan is conducted through sentinel ospitals, clinics, and pharmacies and through manufacturers. Surveillance data on JE vaccine E come principally from the manufacturer (Biken and others). Few neurological complications emporally related to JE vaccination were reported but denominators of vaccinees were not vailable in all years and the sensitivity of this passive surveillance system is unknown (Table 4–18).[216, 283,285]

In 1992, two anecdotal cases of temporally-related vaccine-associated acute sseminated encephalomyelitis (ADE) in Japan prompted a survey of 162 Japanese medical stitutions to solicit additional cases.[286] Five more cases spanning 22 years were reported, cluding two with elevated CSF MBP levels.[287] Neither the numerator of cases nor denominator vaccinees were defined rigorously, however, the authors estimated that ADE occurred less equently than one in a million vaccinees. In an unrelated report, three ADE cases (one fatal) mporally related to vaccination were reported in Korea in 1994 and one in 1996, also fatal. An ldditional fatal case of acute encephalopathy occurred in a 15 year old girl who received her nth dose of JE vaccine and her third dose of hantaviral vaccine (also made in mouse brain) four d two weeks respectively before onset of stupor and seizures (YM Sohn, unpublished servations).

An additional report of vaccination associated ADE cases in Danish travelers, unprompted previous reports from Japan and Korea, suggests that the issue of neurological complications ould be reinvestigated.[288] After a vaccinee developed ADE in 1995, a review of the national tabase disclosed two similar temporally related cases in 1983 and 1989, all in adults. Because vaccine distribution in Denmark is controlled, the denominator of vaccinees and a rate for the verse event could be estimated. The rate of temporally-related ADE, one in 50-75,000 ccinees, is far above previous estimates of all neurological complications and in the same ge as JE incidence in countries where the disease is endemic.

The significance of anecdotal reports from Asia is difficult to interpret, since systematic a for children are unavailable. The incidence of serious vaccine-related neurological nplications, if any can be shown, probably is low because no clear association has emerged ing the more than three decades when the vaccine has been used. Notwithstanding this pression, recent anecdotal reports and the high rate of serious events in the Danish study, gests the need for a controlled study in routinely vaccinated children. In Korea, where no urally acquired JE case has occurred in recent years, public objections to the vaccine have n raised, citing greater risk from the vaccine than from the disease itself.

Although the bovine spongiform encephalopathy outbreak has raised concern over the ential for contamination of biologicals with prions from animal sources, there has been little cussion about risks of the JE vaccine mouse brain substrate. Factors mitigating against such sk are the low, if any, natural incidence of a mouse transmissible spongiform encephalopathy , vaccine purification process which removes certain proteins from the final product and the cies barrier. In the absence of a naturally occurring murine spongiform encephalopathy, the cipal concern is comixing of mice designated for vaccine production with mice infected in a earch project. Although this seems unlikely, mice used in vaccine production are supplied by iple subcontractors whose facilities may be difficult to monitor. The vaccine formalin

inactivation process does not inactivate and, potentially, could stabilize contaminating prions. On balance, it seems highly unlikely that the vaccine poses a risk for transmission of a spongiform encephalopathy agent.

Hypersensitivity reactions Vaccine-related allergic AE, not reported previously from Asia, were recognized after 1989 in Australia and several European and North American countries as the vaccine became used widely in travelers.[181,182,291-294] Hypersensitivity reactions have consisted principally of generalized urticaria and/or angioedema which in a few patients, potentially, were life threatening. The reactions generally have responded to oral antihistamines or corticosteroids but in recalcitrant cases have necessitated hospitalization and parenteral steroid therapy. A temporally related death was reported in a man with multiple hypersensitivities who also had received plague vaccine.[182] Numerous lots and different manufacturers have been implicated.[293] In retrospect, allergic side effects including urticaria, angioedema, and moderate dyspnea were observed in recipients of the crude mouse brain vaccine administered on Okinawa in 1945.[200]

An important feature of the reactions is the potential for delayed onset, particularly after a second dose. In a prospective study of 14,249 U.S. Marines, the median interval between immunization and onset was 18 to 24 hours after the first dose with 74% of reactions occurring within 48 hours.[181,290] Among reactors to a second dose, there was a greater delay with a median interval of 96 hours and a range of 20-336 hours. Reactions have developed after a second or third dose when previous doses were given uneventfully. A nested case-control study found an elevated risk with history of various allergic disorders, e.g.: urticaria, OR 11.4, (95%CI 2.4-62.1); allergic rhinitis, OR 9.2 (95% CI 2.8–23.1); asthma and/or rhinitis, OR 6.5 (95% CI 2.1-20.8) and any allergy, OR 5.7 (95% CI1.8-18.1).[182] Another small study also implicated alcohol consumption and receipt of another vaccine 1-9 days previously, as opposed to simultaneously, as risk factors.[295]

Reported rates have varied according to the approach to ascertainment (Table 24–19). Recent prospective or retrospective studies have found risk of an allergic AE, usually defined as objective urticaria and/or angioedema, in the range of 18-64/10,000 vaccinees. [290,295-298] A cluster of two deaths due to anaphylactic shock in children receiving JE vaccine were reported in Korea in 1994. In a follow-up study to measure incidence of JE vaccine-related adverse events, one case of anaphylactic shock with syncope and collapse, three cases of generalized urticaria and three with severe erythema were found in 15,487 Korean children immunized from 15 May to 30 June 1995. The rate of 0.03% was lower than that observed in adult travellers which could reflect either biological differences in reactivity or the sensitivity of surveillance (Sohn YM, unpublished observations).

Although the pathogenesis of the hypersensitivity reactions is not proven, in three Japanese children experiencing systemic reactions, IgE antibodies to gelatin were demonstrated, suggesting that gelatin, added as a vaccine stabilizer, may be a provoking antigen.[299] A similar syndrome has been described in recipients of diploid cell–derived rabies vaccine in whom symptoms developed after a delay of as long as 1 week after booster immunization.[300] Immunological studies demonstrated IgE antibodies to human albumin, added to the vaccine as a stabilizer and chemically altered by the inactivating agent, (-propionolactone.[301] Allergic reactions in recipients of crude mouse brain vaccine in Okinawa were attributed to formalin-altered proteins.

Inactivated Primary Hamster Kidney Cell–Derived Japanese Encephalitis Vaccine

Few adverse reactions have been reported in connection with the P-3 inactivated vaccine.

Local reactions, including swelling at the injection site, are observed in about 4% of vaccinees, and mild systemic symptoms, such as headache and dizziness, are reported by fewer than 1% of vaccinees. Fever above 38°C previously was a complication in 12% of vaccinees, but with a reduction of bovine serum in the currently formulated vaccine, febrile reactions have been halved. An urticarial allergic reaction was observed in only one of nearly 15,000 vaccinees surveyed.[274]

Live-Attenuated Japanese Encephalitis Vaccine

An estimated hundred million children have been immunized with the live-attenuated vaccine without apparent complication. Clinical monitoring of experimentally immunized subjects has documented the absence of local or systemic symptoms after immunization, specifically, headache and symptoms that might be associated with neuroinvasive infection were not observed. Likewise, fever and signs and symptoms of systemic infection have not been observed after immunization. In a study of 867 children in which fever was monitored over a 21-day period after immunization, temperatures above 37.6°C were recorded in fewer than 0.5% of vaccinees and fever-onset days were distributed throughout the observation interval, mitigating against a vaccine-related febrile illness after a specific incubation period. In the same study, symptoms were recorded from 588,512 other vaccinees, fever was reported in 0.046% of subjects, rash in 0.01%, dizziness in 0.0003%, and nausea in 0.0003%, however, these rates are difficult to interpret in the absence of similar observations in controls.[238,302]

A block randomized cohort study of 13,266 vaccinated and 12,951 unvaccinated 1-2 year old children, followed prospectively for 30 days, was reassuring in confirming the vaccine's safety. No cases of encephalitis or meningitis were detected in either group and rates of hospitalization, new onset of seizures, fever lasting more than three days, and allergic, respiratory and gastrointestinal symptoms were similar in the two groups. The observations excluded a vaccination-related encephalitis risk above 1 in 3,400.[303]

The rates of clinical encephalitis among children vaccinated in field trials (see Table 24–16) provide additional reassurance that SA-14-14-2 virus does not itself cause encephalitis at a detectable rate. Rates of clinical encephalitis in children receiving SA-14-14-2 vaccine, 1.16–6.75/100,000 are lower than reported population based incidence rates of childhood encephalitis (15-30/100,000).

No observations on the vaccine's safety in pregnancy, in immunocompromised persons and specifically in those with HIV infection, have been reported.

Indications for Immunization

Endemic Areas

In rural areas of Asia, intense JE virus transmission in the enzootic cycle leads to a high risk of exposure at an early age. Universal primary immunization is indicated for children between 1 and 2 years of age. The peak risk of infection is in children between 1 and 4 years of age, which may reflect the protective effects of maternal immunity and patterns of outdoor activity that place young children at risk. However, cases occur in children through the first decade of life and in most areas with risk of enzootic transmission, immunity should be maintained by boosters through age 10.

Although incidence may vary regionally in countries at risk, universal childhood immunization is desirable because even in economically advanced countries, viral transmission cannot be eliminated and, the cumulative risk of acquiring the illness over a lifetime of exposure

probably justifies universal protection. Furthermore, conditions leading to epidemic transmission are unpredictable, and at intervals, outbreaks may lead to large numbers of cases even in urban areas. Hong Kong and Singapore may be special cases where despite the absence of a national immunization policy, the possibility of enzootic viral transmission is limited by the exclusively urban environment.[174] For the most part, stepwise implementation of national JE vaccination programs, initially in epidemic foci and in areas with hyperendemic transmission, has been necessary because of economic considerations.[18]

Expatriates

JE vaccine is recommended for expatriates whose principal residence is in an area where JE is endemic or epidemic. (For purposes of this discussion, *expatriates* are defined as residents through a transmission season.) Risk of acquiring JE among expatriates is variable and depends principally on the specific location of intended residence, housing conditions, nature of activities, and the possibility of unanticipated exposure to high-risk areas (see subsequent section). Risk varies regionally and within specific countries. Viral transmission is seasonal in most areas and can fluctuate from year to year in a given location. Figure 24–1 and Table 24–20 summarize and extrapolate available data on locations and seasonality of risk by country. Patterns of viral transmission may change, and physicians and travelers are cautioned to consult public health officials for current data and trends.

Travelers

Japanese encephalitis vaccine is recommended for selected travelers to Asia and should not be considered a routine immunization. Risk of acquiring JE during travel is extremely low (see previous discussion), and the vast majority of visitors to Asia on business or in tours are at low risk and need not be immunized. In addition, the vaccine is costly; the "average wholesale price" (AWP) of three doses in the United States is $147. Because JE viral transmission is confined to certain seasons and occurs principally in rural areas, only visitors with such a travel itinerary have a high risk of acquiring the disease. Travelers and their physicians should weigh individual risk factors and disease risk in the area and season of anticipated travel in light of the potential for vaccine side effects (see Fig. 24–1 and Tables 24–4 and 24–20).[189, 189,292,304]

Immunization is recommended for visitors to epidemic or endemic areas during the transmission season, especially when they have an extended period of exposure (more than 30 days) or they are at high risk of exposure to vectors because of the nature of activities or housing. For example, bicyclists on tours and workers on field projects in rural areas may have greater outdoor exposure to vector mosquitoes. In addition, advanced age and pregnancy may affect risk and outcome of JE. Repellents and other protective measures are recommended in any case because other vector-borne diseases may be transmitted in the same areas. General precautions are especially important to travelers in whom vaccine is contraindicated, who are unable to complete immunization because of departures on short notice, or who do not choose to be immunized because their visits to high-risk areas are brief or carry an equivocal risk.

Because allergic reactions to mouse brain–derived JE vaccine may be delayed for 1 week after immunization, and to allow protective antibody levels to develop, vaccinees ideally should defer travel until 7 days after receiving the last vaccine dose. Travelers should remain in areas accessible to medical care for 7 days after immunization.

Research Laboratory Workers

Japanese encephalitis virus has been transmitted in 22 laboratory-acquired cases, principally in research settings, where infectious JE virus was used.[305] Infection can be transmitted by percutaneous or mucous membrane exposures and potentially by aerosols, especially from preparations containing high viral concentrations, which occur during viral purification. Immunization presumably protects against percutaneous exposures; however, it is unknown whether vaccine-derived immunity, especially from inactivated vaccine, protects against aerosol infection. Immunization is advised for all research laboratory personnel who potentially may be exposed to field or virulent strains of the virus.[292] Although no formal biosafety recommendations have been issued for work with the attenuated vaccine SA14-14-2 strain, sufficient data are available on its attenuation that immunized workers should be permitted to handle that virus under BSL-2 conditions, paralleling recommendations for the attenuated vaccine strains of YF, Junin, Rift valley fever, chikungunya and Venezuelan equine encephalitis.[292]

Contraindications to Immunization

Mouse brain–derived JE vaccine is contraindicated in persons who have had an allergic reaction to the vaccine or to other rodent-derived products, including previous doses of JE vaccine. Other biologicals made in rodent tissue include vaccines against the hantaviral agents of hemorrhagic fever with renal syndrome, Hantaan and Seoul viruses, products derived from Chinese hamster ovary cells and murine monoclonal antibodies. Hantaan virus vaccine made in mouse brain and purified by methods similar to those used in JE vaccine manufacture is produced in Korea and is under evaluation in China. A hantaviral vaccine produced in primary gerbil (*Meriones unguiculatus*) kidney cells also has limited distribution in China. Yellow fever vaccine made from the French neurotropic strain previously was produced in mouse brain, but production was discontinued in 1982.

Anecdotal reports of ADE occurring in temporal relationship to vaccination suggest that the mouse brain derived vaccine should not be used in persons who have recovered from ADE, GBS or who have multiple sclerosis or other demyelinating disorders.

Hypersensitivity reactions to mouse brain–derived JE vaccine are more common in persons with allergic conditions, such as asthma, allergic rhinitis, drug or hymenoptera venom sensitivity, and food allergy (especially to gelatin containing foods - see earlier discussion). If these persons are offered JE vaccine, they should be advised of their potential for vaccine-related angioedema and generalized urticaria. Hypersensitivity to a protein found in mouse urine is common in animal caretakers and certain laboratorians. It is unknown whether this sensitivity carries a specific risk in recipients of JE vaccine. Vaccinees also should be warned of a potential interaction with alcohol consumption.

There are no specific contraindications to the use of PHK–derived inactivated JE vaccine except history of allergic reaction to a previous dose.

Japanese encephalitis vaccines pose a theoretical risk to the developing fetus. No adverse outcomes of pregnancy have been associated directly with JE vaccine. Travelers and their physicians must balance the theoretical risks of JE vaccine in pregnancy against the potential risks of acquiring JE and the adverse outcome of the disease.

There are few data on the safety and efficacy of inactivated JE vaccines in immunocompromised persons. A small study of children with various chronic diseases, including some oncology patients, disclosed no difference in immunogenicity or reactogenicity in recipients of mouse brain–derived vaccine.[267] Infants vertically infected with HIV responded less well to the vaccine (see above) but no unusual adverse events were recorded.[266]

Live-attenuated JE vaccine potentially carries an additional risk in pregnancy and in immunocompromised patients. Although experimental data suggest that JE SA14-14-2 virus may not be neurotropic in immunosuppressed animals, there are no data on the vaccine's safety in immunocompromised persons and specifically, in HIV infected persons. When JE vaccine must be given to pregnant women or to immunocompromised patients, available inactivated JE vaccine should be used rather than live vaccine.

PUBLIC HEALTH CONSIDERATIONS

Although a secular trend toward declining JE incidence has been observed with widespread use of JE vaccine, coincident socioeconomic changes also may have contributed to falling disease incidence (Fig. 24–19). In Thailand, for example, encephalitis incidence had begun a steady decline since the mid 1970's, nearly two decades before the national JE immunization program was instituted in 1990 (Figure 24-5) and in Singapore, reductions in disease incidence and viral transmission have been attributed solely to factors other than vaccination (see above).[13,174] The most important have been improved agricultural productivity and increasing urbanization, resulting in fewer rural dwellers at risk; a decline in land area under rice cultivation; and increased use of agricultural pesticides, which have reduced numbers of vector mosquitoes (Figs. 24–20).[306] Although pig inventories actually have increased, changes in husbandry practices and especially centralized rearing probably has resulted in an overall reduction of infected vectors where people are active. Improvements in the general standard of living and, in specific locations, vector control programs, have further reduced risk of exposure and infection.

Observations from the PRC, where development has been less extensive, are somewhat clearer in demonstrating the impact of immunization. JE incidence rates in Beijing and in other areas of China where high immunization rates are maintained have declined dramatically and have remained low (Fig. 24–13,21 and Table 24–21).[10,194] Although vaccine coverage is high in cities and in prosperous districts, coverage remains low in many rural locations, often in the very places with greatest risk. The principal barriers to immunization include the cost of the vaccine, which must be borne by families since JE vaccine is not government subsidized as a childhood vaccine, and inaccessibility to the health care system.

As a zoonotic disease with natural viral reservoirs, JE never can be eliminated. Although its transmission can be modulated by the factors mentioned above, these approaches alone or in combination cannot be relied upon to reduce disease incidence as effectively as human vaccination. The successful control of JE by universal immunization in at least three countries in the region suggests that an extension of these efforts throughout the continent could lead to the near elimination of the disease. However, for all of the approved vaccines, unresolved issues potentially limit their acceptability as a solution for region-wide control of the disease.

The inactivated mouse brain-derived vaccine is troubled by safety and other issues. Moreover, the vaccine's 91% efficacy when extrapolated to the entire cohort of children <15 years in Asia, approximately one billion children, yields an absolute number of primary vaccine failures of questionable acceptability. Assuming a JE incidence rate of 5/10,000 in children <15, approximately half a million cases would occur in the absence of any immunization. If every child was immunized but only 91% was protected, 45,000 residual cases due to primary vaccine failure would occur annually. Although additional booster doses presumably would improve efficacy, the strategy also would lead to increased costs to a vaccine that already is considered costly and of marginal cost-benefit.

A study of the vaccine's benefits and costs in Thailand showed that a national

immunization program of 18 month old children had an effectiveness of $15,715/case prevented and a benefit/cost ratio of 4.6, at the current Thai domestic production cost of $2.16 for two 0.5 ml doses. A sensitivity analysis based on varying JE incidence rates, showed that the program no longer was economical (where the ratio fell below one) at an incidence rate of 3/100,000. In less developed countries where the prevention of lost productivity would yield lower savings, national vaccination programs would be uneconomical at higher incidence rates. Whether vaccine cost could be reduced further by economies of scale is uncertain since, unlike viral vaccines produced in cell cultures, scaling up production involves considerable labor in the rearing, inoculation, and harvesting of mice as well as an extensive purification process.

The SA14-14-2 vaccine, produced under government subsidy in China, "costs" $0.03 per dose; however, under internationally accepted manufacturing standards its estimated cost per dose will be in the same range as the inactivated mouse brain vaccine. Fewer doses are required for long-term protection however, reducing the overall costs per child protected. The vaccine is under consideration for licensure in Korea and if approved, may be manufactured for international distribution in the future. The principal concerns for its broader acceptance are the potential for adventitious agents associated with the PHK cell substrate and its safety in areas of Asia where HIV infections are prevalent.

History of the live attenuated Japanese encephalitis vaccine strain SA-14-14-2

The Development of Live-attenuated JE Vaccine (SA14-14-2)

Attenuated JE viral strains have been sought by passaging wild strains serially in various cell culture systems, including Primary hamster kidney cell, chick embryo and mouse skin cells as well as other Asian countries. Loss of neurovirulence in mice, hamsters, and/or pigs initially suggested the possibility of safe use in humans. Workers at the National Institute for Control of Pharmaceutical and Biological Products (NICPBP) in Beijing pursued the attenuation of JE virus. Li and Yu, in 1959, found no loss of neurovirulence of JE virus P3 strain even after 110 passages in chick embryo suspended tissue culture. However, when the SA14-14-2, a low peripheral pathogenic strain isolated in 1954 from culex pipiens larvae from Xian, was serially passed in weanling mice 11 times and in hamster kidney cell culture, a remarkable drop of neurovirulence was found after 20 passages. The virus at the one hundredth passage was then plaqued. Three of nine plaques showed very low neurovirulence (0-0.5 log LD_{50}), of which low neurovirulent plaque 12-1-7 was selected for its stabilized genetic property by Li et al in 1966. By further plaquing and selection, a stable avirulent, 5-3 stain was selected for human trial and its biological properties were studied by Yu et al as a vaccine candidate.

The resulting SA14-5-3 strain no longer reverted to an established criterion of neurovirulence after intracerebral passage in suckling mice while remaining potent in mouse immunization-challenge studies. SA14-5-3 virus did not kill 3-week-old mice by either subcutaneous or direct intracerebral inoculation. Direct intrathalamic and intraspinal inoculation of the virus in monkeys resulted in no mortality or morbidity and a minimal degree of CNS inflammation, limited to areas around the injection sites. Histopathological changes were characterized by perivascular lymphocytic cuffs and focal mononuclear cell infiltration with rare direct neuronal degeneration or necrosis.

SA14-5-3 vaccine was shown to be safe in humans and field trials in endemic areas disclosed seroconversion rates greater than 85%. However, rates of only 61% were obtained in subjects from nonendemic areas. Expanded field trials in southern China, involving more than 200,000 immunized children, confirmed the vaccine's safety and yielded efficacies ranging from 88 to 96% over 5 years. However, the vaccine's poor immunogenicity in flavivirus naive subjects from nonendemic areas suggested that SA14-5-3 virus, like previous live JE virus candidate vaccines, had been overattenuated and did not replicate uniformly in humans. To increase immunogenicity, SA14-5-3 virus was serially passaged five times by subcutaneous inoculation of suckling mice, using skin, subcutaneous tissue and local peripheral lymph nodes as the passage material. After plaque selection and cloning twice in PHK cells, the SA14-14-2 strain was obtained. SA14-14-2 virus was equally attenuated but more immunogenic in mice, pigs and humans, producing seroconversion rates greater than 90% in nonimmune subjects.

The reduced neurovirulence of the SA14-14-2 strain was confirmed in three week old mice and monkeys. Compared with the parent SA14 strain, which killed weanling mice by subcutaneous or intracerebral inoculation with LD_{50}s in the range of $10^{5.5}$ to $10^{8.3}$ per ml, respectively, SA14-14-2 virus produced no mortality and only minor clinical signs in a few intracerebrally inoculated animals. Combined intrathalamic and intraspinal inoculation of rhesus monkeys produced no clinical illness and only minor inflammatory reactions in the substantia nigra and cervical spinal cord. Mice were more sensitive than monkeys to intracerebral infection, with some animals showing mild neuronal lesions in the cerebral cortex, hippocampus and/or basal ganglia. Compared with histopathological lesions produced by the parent SA14 virus, the inflammatory reaction to SA14-14-2 virus was greater and neuronal necrosis was significantly less. In five week old mice inoculated intracerebrally with the virus pair, ultrastructural studies showed the parent virus produced cytopathological changes in the majority of neurons, particularly in the rough endoplasmic reticulum and Golgi apparatus of the neuronal secretory system, while it could not be confirmed that the vaccine strain replicated at all and neurons appeared normal.

Further evidence of the strain's reduced neurotropism comes from experimental studies in athymic nude mice. No deaths or histopathological abnormalities were observed after intraperitoneal or subcutaneous inoculation of a viral dose greater than 10^7 TCID50 and virus could not be recovered from brain tissue. Although cyclophosphamide increases susceptibility of mice (and monkeys, as discussed earlier) to virulent JE virus, immunosuppresion with cyclophosphamide did not lead to encephalitis in mice inoculated peripherally with SA14-14-2 virus. The strain also did not kill intracerebrally inoculated weanling hamsters. Phenotypic characteristics of the vaccine strain (PHK8) such as small plaque size and reduced mouse neurovirulence were stable through at least 10 additional PHK cell culture passages.

Compared with two doses of inactivated P-3 vaccine, a single dose is more immunogenic and potent in protecting mice and guinea pigs against challenge, as measured by survival after intracerebral inoculation or suppression of viremia respectively. Six months after immunization, when neutralizing antibody titers declined to undetectable levels (<1:5), mice receiving attenuated vaccine were protected at higher rates (88%) than mice receiving inactivated vaccine (33%). Adoptive immunity, by transfer of immune spleen cells from immunized mice (50% protection versus 10%), and passive protection from immune serum (80% versus 33%), were better in mice immunized with live vaccine. Induction of cellular immunity also was shown by higher levels of protection in cyclophosphamide suppressed immunized mice (see above). Attenuated vaccine provided more effective protection than inactivated P-3 vaccine against a spectrum of JE strains isolated in China.

Attenuation of SA14-14-2 virus was produced empirically by serial cell culture passage and the underlying molecular basis of its neuroattenuation still is under active investigation. The nucleotide sequence of the neurovirulent parent SA14 virus differs from SA14-14-2 and two other attenuated SA14-2-derived vaccine viruses in only seven amino acid substitutions found in all three attenuated strains. Four were in the envelope protein (E-138, E-176, E-315 and E-439), one in nonstructural protein 2B (NS2B-63), one in NS3 (NS3-105), and one in NS4B (NS4B-106).

Studies of other attenuated JE viral strains have shown the spectrum of mutations associated with phenotypic attenuation. ML-17, a pig vaccine strain derived by serial passage in primary monkey kidney cells, contains six amino acid changes in the protein coding region and one nucleotide change within the 3' noncoding region (nt-10512). (Chang unpublished results) An amino acid change at E-138, also present in SA14-14-2 virus, was shown to be sufficient for mouse neuroattenuation when introduced into a JE cDNA infectious clone. The other five changes are unique in ML-17 virus, E-146, NS3-192, NS4a-72, and NS4B-274 and -315. Only six passages of virulent Nakayama and 826309 viruses in HeLa cells (HeLa p6) resulted in significantly reduced neuroinvasiveness and nerovirulence for mice and altered receptor-binding activity. Nucleotide sequences of their structural protein genes revealed that the viruses differed by eight and nine amino acid mutations, respectively. Attenuated viruses also have been obtained by selecting neutralizing-resistant variants. Attenuation was associated with single base changes resulting in single E protein amino acid changes and was linked with altered early virus-cell interactions but not with replication.

SA14-14-2 virus also is propagated in BHK-21 cells as a swine vaccine which has been shown to protect against JE virus-associated abortions. SA14-14-2 and the 2-8 strain, obtained from further attenuation of the 12-1-7 strain, also are manufactured into effective equine vaccines distributed in China.

An estimated 100 million children have been immunized with the SA14-14-2 live-attenuated vaccine without apparent complication. Clinical monitoring of experimentally immunized subjects has documented the absence of local or systemic symptoms after immunization, specifically, headache and symptoms that might be associated with neuroinvasive infection, and fever and signs and symptoms of systemic infection have not been observed after immunization. In a study of 867 children in which fever was monitored over a 21 -day period after immunization, temperatures above 37.6° C were recorded in fewer than 0.5% of vaccines and fever-onset days were distributed throughout the observation interval, mitigating against a vaccine-related febrile illness after a specific incubation period. In the same study, symptoms were recorded from 588,512 other vaccines, fever was reported in 0.046% of subjects, rash in 0.01 %, dizziness in 0.0003%, and nausea in 0.0003%, however, these rates are difficult to interpret in the absence of similar observations in controls.

A block randomized cohort study of 13,266 vaccinated and 12,951 unvaccinated 1-2 year old children, followed prospectively for 30 days, was reassuring in confirming the vaccine's safety. No cases of encephalitis or meningitis were detected in either group and rates of hospitalization, new onset of seizures, fever lasting more than three days, and allergic, respiratory and gastrointestinal symptoms were similar in the two groups. The observations excluded a vaccination-related encephalitis risk above 1 in 3,400.

The rates of clinical encephalitis among children vaccinated in field trials provide additional reassurance that SA-14-14-2 virus does not itself cause encephalitis at a detectable rate. Rates of clinical encephalitis in children receiving SA-14-14-2 vaccine -- 1.16 to 6.75 per 100,000 -- are lower than reported population based incidence rates of childhood encephalitis (15-30 per100,000).

Several small immunogenicity studies of the SA14-14-2 vaccine have been reported, with variable results. After a single dose, antibody responses are produced in 85 to 100% of nonimune l- to 12-year-old children, with a response gradient that parallels progressive vaccine dilution. Lower seroconversion rates were obtained with vaccine dilutions that had infectious titers less than $10^{6.7}$ $TCID_{50}$ per mL, which has been established as the minimal standard of vaccine infectivity.

Because of variable immune response rates after one dose, SA14-14-2 vaccine is given in a schedule of two doses separated by a year, according to the custom of annual spring campaigns. The immunogenicity of two doses given at intervals of either 1 or 2.5 months was shown in 12- to 15-year-old children. Response rates were similar: 75 to 100% after one dose and 94 to 100% after two doses (two vaccine lots were compared), but there was a trend toward better seroconversion with the longer interval, and GMTs were approximately two-fold higher (65-89 versus 115-158, respectively).

Efficacy trials in children 1 to 10 years old have consistently yielded high protection rates above 98%. In the 1991 Yunnan field study, neither of the two cases in vaccinated children produced serious illness, but three deaths occurred in the unvaccinated cohort, and more than 50% of the remaining cases were considered severe. In the Guizhou study, equally good protection was observed through a second year after a booster dose was given.

A study measuring the effectiveness of the SA14-14-2 vaccine, using case-control methods, disclosed protection levels similar to those estimated by previous efficacy studies. When immunization histories were compared among 56 hospitalized laboratory-confirmed JE cases and 1,299 age-matched village controls, the vaccine's effectiveness was 80% for one dose (95% CI = 44-93%) and 98% for two doses (95% CI = 86-99.6%). Because of uncertainties about the methodological approach of earlier efficacy studies, the consistency of this result with previous estimates was reassuring. Furthermore, effectiveness is a measure of the vaccine's performance under the usual circumstances of health care delivery rather than the artificial conditions of a study, which is additional evidence of the vaccine's robustness.

Regulatory Background

- Market Authorization in China (1988)
- Approval on the safety and effectiveness in Korea (1997)

New Drug Certification

We hereby certify that the following new product, live attenuated Japanese Encephalitis Vaccine produced by Chengdu Institute of Biological Products, China is reviewed by Ministry of Health, China, in accordance with the Chinese Pharmaceutical Law and approved to be met for the Regulation of New Biological Products.

May 24 1998

Ministry of Health, the People's Republic of China

新药证书

编号：（88）卫药证字 S－01号

中国药品生物制品检定所
卫生部成都生物制品研究所

你单位研制的新药流行性乙型脑炎减毒活疫苗，根据《中华人民共和国药品管理法》，经审查，符合我部颁发的《新生物制品审批办法》的规定，特发此证。

中华人民共和国卫生部

1988年5月24日

Certificate for Free Sales and Manufacturing

The Ministry of Health of the People's Republic of China hereby certifies that the following pharmaceutical product is manufactured in Chengdu Institute of Biological Products, Ministry of Health, China and approved by Chinese Ministry of Health.

Product Name: Japanese Encephalitis Vaccine, Live attenuated
Virus Strain: SA14-14-2
Indication: For the Prevention against Japanese Encephalitis
License Number: (90) S-01

We hereby certify that the above product is manufactured by Chengdu Institute of Biological Products, Ministry of Health, China in accordance with GMP as recommended by WHO and is subject to control according to the Revised Chinese Regulation for the Manufacturing and Control of Pharmaceutical Products (1992). We also certify that the product is freely sold in China and can be exported to other countries.

October, 1996
Ministry of Health, the People's Republic of China

中华人民共和国卫生部
MINISTRY OF HEALTH PEOPLE'S REPUBLIC OF CHINA

北京后海北沿44号 100725 电话:4034433 44 HOUHAI BEIYAN BEIJING 100725 TEL:4034433

出口证明函

中华人民共和国卫生部药政管理局特此证明，下列产品是由卫生部成都生物制品研究所生产，并获得中华人民共和国卫生部核发的批准文号。

产品名称：日本脑炎减毒活疫苗

毒　　种：SA14-14-2

适 应 症：预防日本脑炎

批准文号：(90)卫药准字(蓉)S-01号

上述制品是卫生部成都生物制品研究所按中华人民共和国卫生部参照世界卫生组织(WHO)《药品生产质量管理规范》(GMP)标准制定颁布的中华人民共和国《药品生产质量管理规范》(1992年修订)制造的，产品质量符合《中国生物制品规程》的标准要求。

特此证明上述产品允许在市场自由销售。

卫生部药政管理局局长 _____

(签　字)

1996年10月

<2>

Import License of New Drug

For the Clinical Investigation

Pharmaceuticals

Company Classification: Importer

Company Ref No. 549

Product Name: CD. JEVAX

(Japanese Encephalitis Vaccine, Live attenuated) Classification No. 631

Composition: Attached 1

Preparation: Lyophilized powder containing live attenuated Japanese Encephalitis virus;
Pink or reddish transparent liquid preparation when reconstituted

Manufacturing Process: Attached 2

Indication: Attached 3

Dosage and Usage: Attached 3

Contraindication & Precaution: Attached 3

Package Size: 0.5 mL x 1, 5, 10 vials (with 0.7 mL diluent)

Storage & Validity: Valid for 1.5 years at 2- 8 ℃
Avoiding the light in the airtight container

Specification & Testing Method: By the manufacturer's criteria

Manufacturer: Chengdu Institute of Biological Products, Ministry of Public Health

License Condition: Attached

We certify that this product is licensed for the clinical investigation in accordance with the Pharmaceutical Law Clause 34, Art 26 in Republic of Korea

August 11, 1997

Ministry of Health and Welfare, Korea

제 2 호

의약품·의약부외품·의료용구·위생용품제조(수입)품목허가증

전문의약품

업 종: 수입의약품 업허가(수입자확인)번호: 549

제 품 명	씨디.제박스(일본뇌염 생바이러스백신)	분류번호	631
원료약품 및 그 분량	별 첨 1		
성 상	약독 일본뇌염 생바이러스를 함유하는 건조제제로써 용제를 가하였을 때 적색 또는 분홍색의 투명한 액상제제		
제 조 방 법	별 첨 2		
효 능 · 효 과	별 첨 3		
용 법 · 용 량	별 첨 3		
사용상의주의사항	별 첨 3		
포 장 단 위	0.5ml/vial × 1, 5, 10파민(0.7ml 용제첨부) 임상시험용의약품		
저 장 방 법 및 사 용 (유 효) 기 간	밀봉용기, 차광하여 2-8° C에서 냉장보관 유효기간 : 18개월		
기 준 및 시 험 방 법	자가기준에 의함 재심사대상의약품		
제조원(수입의경우)	Chengdu Institute of Biolgical Products, Ministry of Public Health		
허 가 조 건	별 첨		

약사법 제26조(제34조)의 규정에 의하여 위와 같이 허가합니다.

1997 년 8 월 11 일

보건복지부장관 인

<별 첨1>

○ 원 료 의 약 품 분 량

1 바이알 중(용제 0.7㎖로 용해하였을 때)

주 성 분: 약독 일본뇌염 생바이러스 (바이러스주 : SA_{14}-14-2)	(별규)	5.4log PFU 이상 함유
안 정 제: 젤라틴	(약전)	5.0mg
자당	(유에스피)	25mg
사람혈청알부민	(생기)	2.5mg
황산카나마이신	(항기)	0.03mg
황산겐타마이신	(항기)	12 IU
착 색 제: 페놀설폰프탈레인	(약전)	0.003mg
첨부용제: 주사용수	(약전)	0.7ml

<별 첨2>

○ 제 조 방 법

바이러스주

SA_{14}-14-2주 또는 이와 동등하다고 인정되는 바이러스주를 사용하며, 이 사용주에 대하여 시드롯트(Seed Lot)를 설정한다. 바이러스주는 시드롯트로부터 3대 이상 계대하여 사용하여서는 안된다.

동물 및 신장

Syrian 햄스터 또는 이와 동등한 감수성이 있는 햄스터의 신장을 사용한다. 동물은 건강하고 튜베르쿨린 반응이 음성이어야 한다. 또한 부검시 뚜렷한 병변을 인정할 수 없는 동물에서 얻어진 신장만을 사용하여야 한다. 만약 신장 이상이나 젖빛의 복수가 발견되면 폐기처분한다.

배양액

세포배양액은 세포배양에 적합한 EMEM 배지를 사용한다. 세포배양액에는 세포증식인자, 0.002w/v% 이하의 페놀설폰프탈레인 및 필요최소량의 항생물질을 넣을 수 있다. 다만 페니실린이나 β-락탐계 항생물질을 넣어서는 안된다. 세포증식인자로서 이종혈청 또는 그 분획분을 사용하였을 때에는 최종원액 중의 함량은 0.0001w/v% 이하가 되도록 제조과정에서 조작을 하여야 한다.

바이러스의 배양액은 바이러스주 배양에 적합한 EMEM 배지를 사용한다. 바이러스 배양액에는 적당한 안정제 및 필요최소량의 항생물질을 가해준다. 다만, 이종혈청 또는 그 분획분 및 페니실린이나 β-락탐계 항생물질을 넣어서는 안된다.

바이러스 부유액

바이러스 배양에는 바이러스주에 적합한 원대 햄스터 신장세포 배양을 사용한다. 개체별 세포배양으로 바이러스를 34~36℃에서 3-4일간 배양하여 세포에 명확한 병변이 나타날 때 수확한다. 수확한 바이러스 부유액을 모아서 개체별 바이러스 부유액으로 한다. 개체별 바이러스 부유액을 모은 것을 여과전 바이러스 부유액으로 한다. 이 때, 착색제로서 0.002w/v% 이하의 페놀설폰프탈레인, 안정제인 사람혈청알부민 및 필요최소량의 항생물질을 가할 수 있다.

여 과

여과전 바이러스 부유액을 원심분리, 여과로 세포를 제거하고 이를 합쳐서 원액으로 한다. 원액은 원액의 시험을 실시하여야 한다.

최종원액 및 동결건조

원액을 혼합하고 필요하면 희석하여 최종원액을 만든다. 이 때 안정제로 1% 젤라틴과 5% 자당을 가해준다. 이 최종원액을 무균적으로 분병하고 동결건조한다.

<별 첨3>

○ 예측 효능 및 효과

일본뇌염의 예방

○ 예측 용법 및 용량

첨부된 용제에 용해하여 0.5㎖를 피하주사한다.

1차 접종 : 접종개시일
2차 접종 : 1차 접종일로부터 1년 후
3차 접종 : 2차 접종일로부터 4년 후
통상 만 1세 이상의 건강한 아동을 대상으로 1세, 2세, 6세에 접종한다.

○ 사용상의 주의사항

1. 금기

1) 발열이 있는 자 또는 영양장애자
2) 급성전염병에 걸린 자
3) 중이염에 걸린 자
4) 활동성 결핵에 걸린 자
5) 심장질환, 신장질환, 또는 간장질환에 걸린 자로 해당질환이 급성기 또는 증악기 및 활동기에 있는 자
6) 임신한 사실이 명백한 자
7) 알레르기 혹은 경련을 일으킨 적이 있는 자
8) 최근 면역억제 치료를 받았거나 현재 면역억제 치료를 받고 있는 자로 면역기능에 이상을 가져올 우려가 있는 자
9) 카나마이신, 겐타마이신에 대해 과거에 과민성을 나타냈다는 것이 확실시 되는 자
10) 다른 생백신(경구용 폴리오 백신, 홍역 백신, 유행성 이하선염 백신, BCG 백신 등)의 접종을 받은 후 1개월을 경과하지 않은 자

2. 신중투여 대상자

1) 젤라틴 함유제제 또는 젤라틴 함유식품에 대해서에 쇼크, 아나필락시스양 증상(두드러기, 호흡곤란, 입술부종, 후두부종 등)등의 과민증의 병력이 있는 환자

3. 부 반 응

1) 건강한 소아 및 성인에 대하여 본제를 접종 후 임상반응은 거의 인정되지 않는다.

2) 드물게 접종후 아래의 증상이 나타는 경우가 있다. 그러나 이들 증상은 경도이고 통상 접종에서는 약 1.0% 이하로 수일 내에 소실된다.

- 37.5℃ 이상의 체온 상승
- 바이러스성 발진
- 현기증
- 오심

4. 적용상의 주의

1) 접종용 기구는 건열, 고압증기, 자비, Ethyleneoxide 가스 또는 코발트 60에서 방출되는 γ 선으로 멸균하고 실온까지 냉각된 것을 사용한다. 그리고 멸균은 가능한 자비 이외의 방법을 따른다.

2) 본제의 용해에 대해서는 용기의 마개 및 용기 주위를 알코올로 소독한 후 첨부한 용제를 균일하게 용해하고 소요량을 주사기 내에 흡입한다. 이 조작을 함에 있어서 잡균이 흡입되지 않게 주의한다. 특히 마개를 벗기거나 혹은 다른 용기에 옮겨 사용하지 말아야 한다.

3) 본제를 주사할 때 절대 소독제가 백신에 접촉하여서는 안된다.

4) 접종부위는 통상 상완 삼각근으로 하고, 알코올 또는 요오드 팅크로 소독한다.

5) 주사침의 선단이 혈관내로 들어가 있지 않나를 반드시 확인해야 한다.

6) 주사침은 피접종자마다 반드시 교환해서 사용한다.

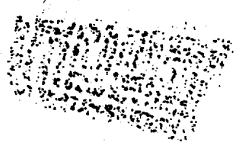
7) 피접종자와 그 보호자에게 접종당일과 그 다음 날은 안정해야 되고, 접종부위를 청결히 유지하고 특히 접종 후 고열, 경련 등의 증상을 나타낼 경우에는 신속하게 의사의 진찰을 받도록 주지해야 한다.

8) 젤라틴 함유제제의 투여로 쇼크, 아나필락시스양 증상(두드러기, 호흡곤란, 입술부종, 후두부종 등)등이 나타났다는 보고가 있으므로 문진을 충분히 하고, 투여후에는 충분히 관찰해야 한다.

5. 취급상의 주의

1) 용해시 내용을 잘 조사하여 침전 및 이물혼입 또는 기타 이상이 인정되거나 용해 전 백신의 색깔이 변한 것을 사용해서는 안된다.

2) 본제의 용해는 접종 전에 실시해서 한 번 용해한 것은 바로 사용한다.



허 가 조 건

1. 임상시험용에 한함.

2. "의약품 등의 안전성·유효성 심사에 관한 규정(보건복지부고시 제1997-49호, '97. 7.31)" 제16조 규정을 준수하고 임상시험용 허가조건 삭제를 위한 변경허가 신청이전까지 식품의약품안전본부장이 인정하는 기준 및 시험방법을 제출할 것.

3. 약사법 제26조의2 및 같은법시행규칙 제30조 제1항 제1호 가목 규정에 의한 재심사 대상품목임.

4. 수입시 다음의 서류를 첨부하여 통관할 것.
   - 생산국의 제조업소에서 발급한 품질관리 적합 성적서.
   - 인혈장 및 혈액제제 생산자가 생산국 정부로부터 허가를 득한 기관이라는 것을 증명하는 서류.
   - 본 제품에 함유되어 있는 인혈장 및 혈액제제 원료가 B형, C형 간염 및 AIDS항체 음성인 공혈자의 혈액으로부터 제조되었음을 입증하는 제조회사 또는 혈장공급 회사에서 발행한 증명서류.

5. 상기 자료를 식품의약품안전본부에 제출하고 등 기관의 AIDS 음성검사를 필할 것.

6. 만일, 정당한 사유없이 상기 조건을 이행하지 아니할 경우에는 본 품목허가를 취소할 수 있다.

Molecular study on attenuation

- Molecular basis of attenuation of neurovirulence of wild-type Japanese encephalitis virus strain SA14
  J Gen Vir 76:409-413, 1995

- Identification of mutations that occurred on the genome of Japanese encephalitis virus during the attenuation process
  Virus Genes 5:2, 95-109, 1991

- Nucleotide sequence of the virulent SA14 strain of Japanese encephalitis virus and its attenuated vaccine derivative, SA14-14-2
  Virology 177, 541-552, 1990

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Journal of General Virology (1995), 76, 409–413. Printed in Great Britain

Molecular basis of attenuation of neurovirulence of wild-type Japanese encephalitis virus strain SA14

Haolin Ni,[1] Gwong-Jen J. Chang,[2] Hong Xie,[1] Dennis W. Trent[2]† and Alan D. T. Barrett[1]*

[1] *Department of Pathology F-05, University of Texas Medical Branch, Galveston, TX 77555-0605, and*
[2] *Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Public Health Service, US Department of Health and Human Sciences, Fort Collins, CO 80522-2087, USA*

To identify the molecular determinants for attenuation of wild-type Japanese encephalitis (JE) virus strain SA14, the RNA genome of wild-type strain SA14 and its attenuated vaccine virus SA14-2-8 were reverse transcribed, amplified by PCR and sequenced. Comparison of the nucleotide sequence of SA14-2-8 vaccine virus with virulent parent SA14 virus and with two other attenuated vaccine viruses derived from SA14 virus (SA14-14-2/PHK and SA14-14-2/PDK) revealed only seven amino acids in the virulent parent SA14 had been substituted in all three attenuated vaccines. Four were in the envelope (E) protein (E-138, E-176, E-315 and E-439), one in non-structural protein 2B (NS2B-63), one in NS3 (NS3-105), and one in NS4B (NS4B-106). The substitutions at E-315 and E-439 arose due to correction of the SA14/CDC sequence published previously by Nitayaphan *et al.* (*Virology* 177, 541–552, 1990). The mutations in NS2B and NS3 are in functional domains of the trypsin-like serine protease. Attenuation of SA14 virus may therefore, in part, be due to alterations in viral protease activity, which could affect replication of the virus.

Japanese encephalitis (JE) is the most common epidemic viral encephalitis in the world today (Gunakasem *et al.*, 1981). There are approximately 50000 clinical cases of JE each year; 25% are fatal (Huang, 1982; Monath, 1990). JE virus, like other flaviviruses, has a positive-sense ssRNA genome approximately 11 kb in length, which encodes three structural proteins [core (C), membrane (M) and envelope (E)] and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5; Brinton, 1986).

The most promising live attenuated JE virus vaccine to control JE is the Chinese SA14-14-2 virus derived from the wild-type strain SA14 (Yu *et al.*, 1973). The safety and efficacy of this attenuated vaccine have been confirmed in human vaccinees (Ao *et al.*, 1983; Yu *et al.*, 1981, 1988). Passage histories of the SA14 attenuated vaccine viruses have been described previously (Ni *et al.*, 1994). Briefly, the first attenuated variant, 12-1-7, was obtained after 100 passages of SA14 in primary hamster kidney (PHK) cells. Vaccine virus SA14-2-8 was derived following treatment of the 12-1-7 virus with ultraviolet irradiation and plaque purification in PHK cells, while vaccine virus SA14-5-3 was derived from 12-1-7 virus by additional plaque purification passages in PHK cells. SA14-14-2/PHK virus was derived by passage of SA14-5-3 virus in suckling mice and plaque purification in PHK cells. SA14-14-2/PDK virus was derived by nine passages of SA14-14-2/PHK virus in primary dog kidney (PDK) cells.

The molecular basis of JE virus attenuation has not been elucidated, although the entire genomes of both virulent parent SA14 and attenuated vaccine clones, SA14-14-2/PHK (Aihara *et al.*, 1991) and SA14-14-2/PDK (Nitayaphan *et al.*, 1990), have been sequenced and compared. Nucleotide sequences of SA14 published by the two groups are not identical, and nucleotide differences were identified throughout the genome between parent and the two attenuated viruses. Aihara *et al.* (1991) identified 57 nucleotide changes coding for 24 amino acid substitutions between SA14 (which we term SA14/JAP) and SA14-14-2/PHK. Nitayaphan *et al.* (1990) reported 45 nucleotide changes coding for 15 amino acid substitutions between SA14 (which we term SA14/CDC) and SA14-14-2/PDK viruses.

* Author for correspondence. Fax +1 409 772 3606. e-mail A.BARRETT@BEACH.UTMB.EDU

† Present address: Center for Biologics Evaluation and Research, Food and Drug Administration, Building 29A, Room 1A23, Bethesda, MD 20892, USA.

The nucleotide sequence data reported here have been deposited with GenBank and assigned the accession numbers U15763 (SA14-2-8) and U14163 (SA14/USA).

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Table 1. *Nucleotide and amino acid differences of the entire genomic sequences between SA14/USA, SA14/CDC, SA14/JAP and three vaccine virus derivatives*

		Nucleotide					
	Strain	SA14/USA	SA14/JAP*	SA14/CDC†	SA14-14-2/PHK*	SA14-14-2/PDK†	SA14-2-8
Amino acid	SA14/USA		15	18	60	56	39
	SA14/JAP	7		15	57	58	42
	SA14/CDC	9	9		64	47	45
	SA14-14-2/PHK	27	24	17		21	60
	SA14-14-2/PDK	22	22	17	8		56
	SA14-2-8	18	19	23	29	24	

* Aihara *et al.* (1991).
† Nitayaphan *et al.* (1990).

To help to identify the molecular determinants of attenuation of JE virus strain SA14, we determined the nucleotide sequence of the 5′ non-coding region and structural protein genes of attenuated vaccine viruses SA14-5-3 and SA14-2-8. The same region of the parental SA14 virus (which we termed SA14/USA) was also cloned and sequenced (Ni *et al.*, 1994). We now report a comparison of the entire genomes of the SA14-2-8 vaccine virus and the parental virus SA14/USA and identify mutations in the viral serine protease.

The RNA genomes of SA14/USA and SA14-2-8 viruses were reverse transcribed and the resulting cDNA amplified, cloned and sequenced as described by Ni *et al.* (1994). The oligonucleotide primers were synthesized based on published SA14 genomic sequence data (Nitayaphan *et al.*, 1990). Nucleotide changes in SA14/USA and SA14-2-8 viruses were confirmed by sequencing different PCR products of the same region. Nucleotides 1863–2463 of SA14/CDC virus were sequenced again. A summary of the nucleotide and amino acid differences of the entire genome between the three vaccine viruses (SA14-2-8, SA14-14-2/PHK and SA14-14-2/PDK) and three sequences of SA14 virus (SA14/JAP, SA14/CDC and SA14/USA) are presented in Table 1. In comparison to the parent SA14 virus, the genome of SA14-2-8 virus had fewer nucleotide and amino acid differences than did SA14-14-2/PHK and SA14-14-2/PDK viruses. This was probably due to different passage histories and fewer passages of SA14 virus to derive the SA14-2-8 virus compared to SA14-14-2 viruses (see Ni *et al.*, 1994). Amino acid differences in the coding region and nucleotide differences in the 5′ and 3′ non-coding regions of SA14 virus and its attenuated vaccine viruses are shown in Table 2.

NS protein genes of the three wild-type SA14 viruses with different passage histories differed by 29 nucleotides encoding 11 substituted amino acids (Tables 1 and 2). These differences may reflect the passage histories of the different SA14 viruses (Ni *et al.*, 1994). The SA14/USA JE virus seed was a mouse brain preparation of SA14 virus while the SA14/CDC JE virus was derived from the same mouse brain preparation containing SA14/USA virus following three passages in PDK cell culture (Eckels *et al.*, 1988; Nitayaphan *et al.*, 1990). The SA14/JAP virus was derived by plaque purification of SA14 virus in BHK-21 cells (Aihara *et al.*, 1991). Four of the amino acid changes are found in the NS1 protein at positions NS1-292 (Ser or Gly), NS1-339 (Arg or Met), NS1-354 (Asn or Lys) and NS1-392 (Ala or Val). Other changes are located at positions NS2A-46 (Val or Ile), NS2B-102 (Thr or Met), NS3-215 (Ala or Val), NS4A-49 (Arg or Lys), NS5-328 (Lys or Glu), NS5-644 (Asn or Thr) and NS5-731 (Gly or Asp) (Table 2). Amino acid changes at NS2B-102 and NS4A-49 of SA14/USA are unique changes compared to the SA14 vaccine viruses and other wild type JE viruses (Table 2).

Three common amino acids have been substituted in the NS protein genes of the three JE attenuated vaccine viruses derived from the parent SA14 virus, at positions NS2B-63, NS3-105 and NS4B-106 (Table 2). In SA14-14-2/PHK and SA14-14-2/PDK viruses, the glutamic acid found at position NS2B-63 in SA14 viruses was substituted by asparagine, while in the SA14-2-8 virus it was replaced by glycine. The NS3-105 substitution was present in all of the vaccine viruses where alanine in the parental SA14 viruses was substituted for a glycine in the three vaccine strains. The isoleucine at position NS4B-106 of the SA14 viruses was substituted by valine in all vaccine viruses. Furthermore, amino acids at positions NS2B-63, NS3-105 and NS4B-106 in all three of the vaccine viruses were different from those of other published wild type viruses (Table 2).

Analysis of the E protein gene of wild-type and vaccine viruses showed that the SA14/CDC sequence, reported by Nitayaphan *et al.* (1990), was the same as the vaccine strains at E-315 and E-439 (Ni *et al.*, 1994). To verify this observation, cDNA to this region of the SA14/CDC virus genome was prepared by RT–PCR and sequenced. This revealed that, contrary to the sequence published by Nitayaphan *et al.* (1990), the two amino acids at positions

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Table 2. *Comparison of amino acid differences in proteins and nucleotide differences in 5′ and 3′ non-coding regions between JE wild-type and vaccine viruses*[*]

Position nt	aa	SA14/ USA[a]	SA14/ CDC[b]	SA14/ JAP[c]	SA14-14-2 PHK[c]	SA14-14-2 PDK[d]	SA14-2-8[e]	JaOAr S982[f]	Beijing-1[f]
39	5′NCR	U	-	-	A	A	-	A	A
292	C-65	Leu	-	-	Ser	Ser	-	-	-
1296	E-107	Leu	-	-	Phe	Phe	-	-	-
1354	E-126	Ile	-	-	-	-	Thr	-	-
1360	E-128	Arg	-	-	-	-	Lys	-	-
1389	E-138	Glu	Glu	Glu	Lys	Lys	Lys	Glu	Glu
1503	E-176	Ile	Ile	Ile	Val	Val	Val	Ile	Ile
1506	E-177	Thr	-	-	Ala	-	-	-	-
1512	E-179	Lys	-	-	-	-	Glu	-	-
1704	E-243	Glu	-	-	-	Lys	-	-	-
1708	E-244	Glu	Gly	-	Gly	Gly	Gly	-	Gly
1769	E-264	Gln	Gln	-	His	-	-	-	-
1813	E-279	Lys	-	-	Met	Met	-	-	-
1921	E-315	Ala	Ala	Ala	Val	Val	Val	Ala	Ala
1977	E-334	Pro	-	Ser	-	-	-	-	-
2293	E-439	Lys	Lys	Lys	Arg	Arg	Arg	Lys	Lys
3184	NS1-236	Val	-	-	-	-	Ala	-	-
3351	NS1-292	Ser	-	Gly	-	-	Gly	Gly	Gly
3493	NS1-339	Arg	Met	-	Met	Met	-	-	-
3528	NS1-351	Asp	-	-	His	-	-	-	Gly
3535	NS1-353	Phe	-	-	-	-	Ser	-	-
3539	NS1-354	Asn	Lys	-	Lys	Lys	-	-	-
3652	NS1-392	Ala	Val	-	Val	Val	-	-	-
3849	NS2A-46	Val	Ile	-	-	-	-	-	-
4402-3	NS2B-63	Glu	Glu	Glu	Asp	Asp	Gly	Glu	Glu
4408	NS2B-65	Asp	-	-	Gly	Gly	-	-	-
4519	NS2B-102	Thr	Met	Met	Met	Met	Met	Met	Met
4782	NS3-59	Met	-	-	Val	Val	-	-	-
4825	NS3-73	Arg	-	-	Lys	Lys	-	Lys	Lys
4921-2	NS3-105	Ala	Ala	Ala	Gly	Gly	Gly	Ala	Ala
5243	NS3-215	Ala	-	Val	Val	-	-		-
5634	NS3-343	Arg	-	-	Trp	-	-	-	-
66341	NS4A-27	Ile	-	-	Trp	-	-	-	-
6700	NS4A-49	Arg	Lys	Lys	Lys	Lys	Lys	Lys	Lys
7227	NS4B-106	Ile	Ile	Ile	Val	Val	Val	Ile	Ile
7706	NS5-51	Glu	-	-	-	-	Asp	-	-
8658	NS5-328	Lys	Glu	-	-	-	-	-	-
8832	NS5-386	His	His	-	Tyr	Tyr	-	-	-
9603	NS5-643	Glu	-	-	-	-	Lys	-	-
9607	NS5-644	Asn	Thr	-	-	Thr	-	Thr	Typ
9688	NS5-671	Val	-	-	Ala	-	-	-	-
9898	NS5-731	Gly	-	Asp	-	-	-	-	-
10428	3′NCR	U	-	-	C	C	-	-	-
10784	3′NCR	C	-	-	U	-	-	U	-

[*] The nucleotides and amino acids listed are at equivalent positions in each strain of JE virus. Sequences were derived from *a*, Ni *et al.* (1994) (nucleotides 28–2463 only); *b*, Nitayaphan *et al.* (1990) (except nucleotides 1863–2463); *c*, Aihara *et al.* (1991); *d*, Nitayaphan *et al.* (1990); *e*, Sumiyoshi *et al.* (1987); *f*, Hashimoto *et al.* (1988). Dashes indicate the same amino acid or nucleotide as SA14/USA.

E-315 and E-439 were alanine and lysine, as found in all other wild-type JE viruses (Table 2). Therefore, there were a total of four amino acid substitutions in the E protein of the attenuated SA14 viruses: two reported by Ni *et al.* (1994) at E-138 and E-176 and the additional two described here at positions E-315 and E-439.

At position 63 of NS2B, a glutamic acid residue was present in all wild-type mosquito-transmitted flaviviruses that have been sequenced except dengue 4 virus (Falgout *et al.*, 1993). In comparison, two JE vaccine viruses (SA14-14-2/PHK and SA14-14-2/PDK) had an aspartic acid residue, the same amino acid present in dengue virus. Vaccine virus SA14-2-8 has glycine at this positio: Since only one strain of dengue 4 virus has bee sequenced (Mackow *et al.*, 1987), we sequenced tl central region of another strain of dengue 4 virus (703– and found the nucleotide sequence of the two strains : be identical. Thus, dengue 4 virus has an unique amir acid at NS2B-63 compared to all other mosquito-bor flaviviruses analysed to date. The substitution at NS2 63 may be important because the glutamic acid position NS2B-63 of wild-type strains of JE virus is al

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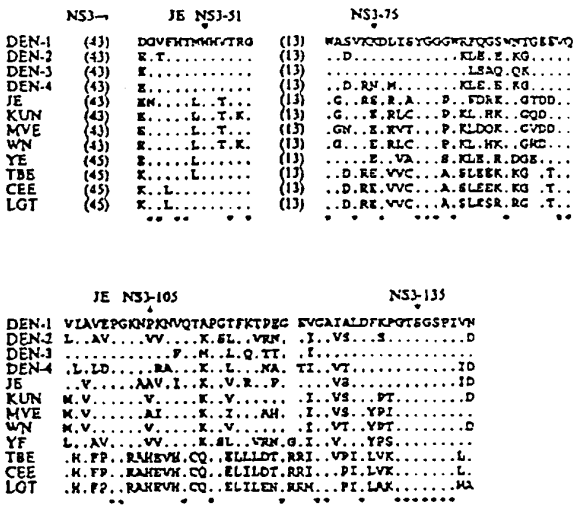

Fig. 1. Alignment of amino acid sequences surrounding the catalytic triad of the serine proteinase and NS3-105 of several important flaviviruses. The numbers in brackets refer to intervening amino acids. Catalytic triad, '▼'; conserved amino acid in the flaviviruses, '*'; amino acid substituted in JE vaccine viruses derived from SA14 virus, '↓'. The viral sequences of DEN-1, DEN-2, DEN-3 and DEN-4 (dengue viruses 1 to 4) are taken from Fu *et al.* (1992), Blok *et al.* (1992), Osatomi & Sumiyoshi (1990) and Falgout *et al.* (1993), respectively; the JE virus sequence is from Sumiyoshi *et al.* (1987); KUN (kunjin virus), from Coia *et al.* (1988); MVE (Murray Valley encephalitis virus), from Dalgarno *et al.* (1986); WN (West Nile virus), from Castle *et al.* (1986); YF (yellow fever virus), from Hahn *et al.* (1987); TBE (tick-borne encephalitis virus), from Pletnev *et al.* (1990); CEE (Central European encephalitis virus, Neudoerfl strain), from Mandl *et al.* (1989); LGT (langat virus), from Iacono-Connors & Schmaljohn (1992).

present in the analogous position of nine other mosquito-borne flaviviruses. Chambers *et al.* (1993), working with yellow fever virus, and Falgout *et al.* (1993), working with dengue 4 virus, found that mutations in this region of NS2B protein reduces or eliminates cleavage efficiency of the virus-encoded serine protease. This suggests that the conserved 'central region' of NS2B has a critical role in the function of the protease.

The protease domain in the N-terminal region of the NS3 protein (Chambers *et al.*, 1990*a*, *b*) of different flaviviruses contains many conserved amino acids (Fig. 1). Wild-type JE and tick-borne encephalitis complex viruses have alanine at NS3-105, while the other mosquito-borne flaviviruses contain asparagine; the JE vaccine viruses have glycine at this position. Since the catalytic triad (NS3-51, NS3-75 and NS3-135; Fig. 1) of the NS3 serine proteinase has not been mutated, its function will not have been eliminated by the change at NS3-105; however, activity of the enzyme could be affected through alteration of the structure of NS3. This proposal is supported by the observation that substitution in the NS3 protein at amino acid position 105 arose because of a double nucleotide change, suggesting that this substitution was selected during the attenuation process. Taken together these data suggest that protease activity may be altered by changes in conformation and/or structure of the NS2B/NS3 complex, which may contribute to attenuation of JE virus.

Alignment of amino acids in NS4B for different flaviviruses shows that amino acid NS4B-106 is valine for all mosquito-transmitted flaviviruses (including JE vaccine viruses) except yellow fever and the wild-type JE viruses, which contain isoleucine. The significance of the valine amino acid substitution in the attenuated phenotype is questionable since valine at this position is strongly conserved among the JE serocomplex viruses.

We have compared the three sequences of the SA14 virus with the sequences of other wild-type JE virus strains and the three attenuated vaccine strains in an attempt to identify substitutions in the SA14 genome associated with attenuation. Since the SA14 vaccine virus strains have received different passages following the isolation of the attenuated clone 12-1-7 virus (Chen & Wang, 1974; Yu *et al.*, 1981; Li, 1986; Eckels *et al.*, 1988), nucleotide and amino acid changes unrelated to attenuation may have resulted. Therefore, a comparison of common nucleotide and/or amino acid differences between SA14 virus and three vaccine derivatives enables the identification of common substitutions that may be responsible for the virus attenuation. Only seven common amino acids were substituted in all three attenuated vaccine viruses compared with parental sequences: four located in the E protein gene at positions E-138, E-176, E-315 and E-439, one at position NS2B-63, another at position NS3-105 and one at position NS4B-106 (Table 2). From these studies we were unable to identify the precise mutations involved in attenuation of neurovirulence of wild-type strain SA14. However, one or more of the seven common amino acid changes may contribute to attenuation of neurovirulence of wild-type strain SA14. Determining whether or not these common amino acid substitutions in the E protein and serine protease complex are directly involved in attenuation of the vaccine viruses will require an analysis of pathogenesis using recombinant viruses.

We thank Kate Ryman for advice with the manuscript.

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Identification of Mutations That Occurred on the Genome of Japanese Encephalitis Virus During the Attenuation Process

SHINOBU AIHARA,[1] RAO CHUNMING,[1,2] YU YONG-XIN,[2] TOMIO LEE,[3] KOJI WATANABE,[3] TOMOYOSHI KOMIYA,[3] HIDEO SUMIYOSHI,[1] HIROSHI HASHIMOTO,[3] AND AKIO NOMOTO[1]

[1]*Department of Microbiology, The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan*
[2]*National Institute for the Control of Pharmaceutical and Biological Products, Beijing, People's Republic of China*
[3]*Department of Virology, The Kitasato Institute, Tokyo, Japan*

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Requests for reprints should be addressed to Akio Nomoto, Department of Microbiology, The Tokyo Metropolitan Institute of Medical Science, Honkomagome, Bunkyo-ku, Tokyo 113, Japan.

Key words: Japanese encephalitis virus, attenuated strain, molecular cloning, nucleotide sequence, mutation site, adaptation

Abstract

The total nucleotide sequences of the genomes of two Japanese encephalitis virus (JEV) strains, the attenuated vaccine strain SA_{14}-14-2 and its parental virulent strain SA_{14}, were determined by using the molecular cloning technique. The sequence analysis revealed that both virion RNA molecules were 10,976 nucleotides long with 95 and 585 flanking bases at the 5′ and 3′ untranslated sequences, respectively. A single, long open reading frame spanning 10,296 nucleotides was observed to encode a polyprotein of 3432 amino acid residues. When these sequences were compared with each other, 57 nucleotide substitutions were found to be scattered all over the genome. Of these, 24 resulted in amino acid changes within viral proteins. Structural proteins C and E contain one and eight amino acid changes, respectively. Of the nonstructural proteins, NS1 contains three, NS2a two, NS2b two, NS3 four, NS4a one, NS4b one, and NS5 two amino acid substitutions. The 5′- and 3′-terminal untranslated regions contain one- and two-point mutations, respectively. These data and comparative studies with other JEV strain genomes provide a molecular basis for investigating attenuation mechanisms of JEV.

Introduction

Japanese encephalitis virus (JEV) is a member of the family Flaviviridae, which includes approximately 60 viruses, many of which are of major concern in human health. Flaviviruses are serologically related to each other and share a similar structural feature. The genome of this small envelope virus is a single, positive-stranded RNA of approximately 11 kb that is capped at the 5′ end and unpolyadenylylated at the 3′ end (1). The viral RNA has a single, long open reading frame (ORF) of approximately 10 kb for the synthesis of a viral polyprotein. The polyprotein is subsequently processed to form viral structural and nonstructural proteins (2–7). Studies on the sequences of RNA and of amino acids in viral proteins have provided a precise viral protein map (2,8–10). To date, complete nucleotide sequences of the genomes of two different JEV strains, JaOArS982 (6) and Beijing-1 (7), have been reported, and the gene order is C-prM-E-NS1-NS2a-NS2b-NS3-NS4a-NS4b-NS5.

Japanese encephalitis (JE) is a widespread viral disease in East, Southeast, and South Asia. JEV that causes JE is transmitted in nature by mosquitoes that preferentially breed in rice fields. The geographic distribution of JE dramatically shifted between 1967 and 1970 (11). In Japan, Korea, and Taiwan, the morbidity rates of this disease have rapidly decreased since 1967. This regional decline has mainly been due to a combination of increased distribution of vaccine and altered agricultural practices. On the other hand, in Vietnam, Thailand, Nepal, and India, epidemic JE has been recognized from 1965 to 1978, and JE still continues to be a significant public health problem in these Asian countries.

A number of cases of JE also occur in most provinces in China (12). Since the revolution in 1949, many studies have been carried out on the epidemiology of JE and the development of the vaccines. Inactivated JE vaccines were prepared from infected primary hamster kidney cell cultures (PHK) (12). Live-attenuated JE vaccines were later prepared for the attainment of higher protection against this disease. The attenuated vaccine strain SA_{14}-14-2 was recognized to be most effective of these (13). This vaccine strain was derived from the virulent virus SA_{14} by multiple passages, mainly through PHK cells (12). In 1987, more than 500,000 children were inoculated with this live vaccine. The results indicated that the attenuated live vaccine was effective in promoting an immune response in recipients, with no side effects. Sometimes, seroconversion rates were more than 90% after administrating only one dose of the attenuated vaccine (13). The live vaccine SA_{14}-14-2 is now being used effectively to prevent JE in China. However, the mechanism that is responsible for the attenuation is totally unknown at present.

As a first effort to give an insight into the molecular basis for the biological differences between the virulent and attenuated JEV strains, we determined the total primary nucleotide sequences of the genomes of the virulent SA_{14} and attenuated SA_{14}-14-2 strains. We report here the complete 10,976-nucleotide sequence

of the JEV genomes and the mutation sites, which were identified by comparing the nucleotide sequences with each other.

Materials and Methods

Cells and viruses

BHK-21 cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 5% calf serum (CS) and 0.3% tryptose phosphate broth (TPB). Monolayer-cultured C6/36 were maintained at 27°C in MEM containing heat-inactivated 10% fetal calf serum (FCS) and seven nonessential amino acids at a concentration of 0.2 mM each (14).

JEV live attenuated vaccine strain SA_{14}-14-2 was isolated by Yu Yong-Xin et al. (15) according to the attenuation processes shown in Table 1. The parental virulent virus SA_{14} was obtained from a pool of larvae of *Culex pipiens* mosquitoes by 11 serial passages in mouse brain.

Plaque assays

To measure virus titers, monolayers of BHK-21 cells in 60-mm plastic dishes were washed twice with MEM containing 0.3% TPB, covered with 0.5 ml of a virus solution (a dilution of the virus stock), and kept at room temperature for 20 min. After incubation at 37°C for 20 min, the cells were washed twice with MEM with 0.3% TPB and then covered with the same medium containing 1% agarose and 5% CS. After 7 days of incubation at 37°C, plaques were visualized by staining cells with 3 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bro-

Table 1. Processes of JEV attenuation

JEV	Attenuation process	Size of plaques[a]
SA_{14} (parent)		Large
$PHKC_{100}$	100 passages of SA_{14} in PHK cells	Large and small
Clone 12-1-7	3 plaque purifications of a small plaque variant of $PHKC_{100}$	Small
Clone 9-7	7 plaque purifications with 1 mouse intraperitoneal passage and 1 mouse subcutaneous passage of clone 12-1-7	Small
Clone 5-3	6 hamster oral passages of clone 9-7, and 2 plaque purifications	Small
Clone 14-2	5 suckling mouse subcutaneous passages followed by 2 plaque purifications	Small

[a] Plaque size was examined using LLC-MK2, chick embryo, or BHK-21 cells.

mide (MTT) (Sigma) in 0.15 M NaCl at 37°C for 1 hr or crystal violet, as previously described (see ref. 19). In some cases, African green monkey kidney cells were also employed to measure virus titers (16).

Virus growth

The seed viruses prepared by plaque purification of SA_{14} and SA_{14}-14-2 viruses in BHK-21 cells were inoculated to microcarrier-cultured C6/36 cells using Cytodex 1 (Pharmacia), and 3 ls of infected fluid were harvested on each day from the second to fifth day of the culture, as described by Sumiyoshi et al. (6), and the viruses derived from SA_{14} and SA_{14}-14-2 were designated SA(V) and SA(A) respectively, and used in this study.

Mouse neurovirulence test

Each mouse (ddY strain) was inoculated with 30 μl of virus solution intracerebrally. Mice were observed every 12 hr for clinical symptoms and death up to 21 days.

Preparation of virion RNA

The infected fluid (3 ls) was filtered through a paper (Toyo filter paper 24CM2) and centrifuged at 8000 rpm for 20 min at 4°C in a Hitachi RR10A rotor. The supernatant was added with polyethylene glycol 6000 and NaCl to final concentrations of 6% and 0.5 M, respectively, kept at 4°C overnight, and centrifuged at 8000 rpm for 20 min at 4°C. The pellet was suspended in 30 ml STE buffer containing 0.1 M NaCl; 10 mM Tris-HCl, pH 7.4; 1 mM EDTA; and centrifuged at 15,000 rpm for 15 min at 4°C in a Beckman type 45Ti rotor. JEV was pelleted from the supernatant by centrifugation at 30,000 rpm for 3 hr at 4°C in a Beckman type 45Ti rotor. The pellet was suspended in a small volume of STE buffer and extracted with $NaDodSO_4$-phenol. Virion RNA was precipitated with ethanol from the aqueous phase.

Molecular cloning and sequencing

Single-stranded cDNA of the JEV genome was synthesized using avian myeloblastosis virus reverse transcriptase and a 16-nucleotide synthetic DNA primer corresponding to the consensus sequence, including the terminal base at the 3' end of JEV genome (6,7). Double-stranded cDNA was then prepared according to the method of Gubler and Hoffman (17) using a cDNA synthesis kit (Amer-

sham). The cDNAs were treated with T4 DNA polymerase to make blunt ends, digested with appropriate restriction enzymes, and then ligated to cloning vector pUC18 or pUC19, which had been treated with the same restriction enzymes as used for digesting cDNAs and/or *Sma*I or *Hin*cII for blunt-end ligations. *Escherichia coli* strain SCS-1 was transformed with the recombinant plasmids, and transformants carrying JEV cDNAs were selected by hybridization using various cDNAs of JaOArS982 (6) and Beijing-1 (7) as probes.

Cloned DNAs were subcloned into the appropriate sites of pUC118 or pUC119, and the nucleotide sequences were determined by the dideoxy termination method (18) using the Sequenase sequencing kit (United States Biochemical Cooperation) with M13 primers or synthetic oligonucleotides corresponding to the appropriate sites of the genome. In some cases, the nucleotide sequences of virion RNAs were directly determined using synthetic oligonucleotides as primers, according to the dideoxy method reported previously (19).

Results

JEV strains and their biological differences

The passage history to obtain JE vaccine strain SA_{14}-14-2 is shown in Table 1. When the parent SA_{14} that showed the large-plaque phenotype was passaged 100 times in PHK cells, small-plaque variants were generated in the virus preparation. After nine plaque purifications in primary-cultured chick embryo (CE) cells and two plaque purifications in PHK cells of a small-plaque variant, an attenuated clone SA_{14}-5-3 was obtained. Five passages of the clone SA_{14}-5-3 in subcutaneous tissue of suckling mice followed by two plaque purifications in PHK cells resulted in the selection of the SA_{14}-14-2 strain. The latter clone was immunogenically superior to the former clone, SA_{14}-5-3 (13). Thus, the attenuated JE vaccine SA_{14}-14-2 is a JEV strain that is adapted to PHK cells, and therefore it grows much more efficiently than the parental virulent SA_{14} in PHK cells (data not shown). However, the SA_{14}-14-2 strain displays smaller plaques in LLC-MK2, CE, and BHK-21 cells than the parent SA_{14} virus (Table 1, Fig. 1).

Both the wild and attenuated strains were plaque purified in BHK cells, and then viruses were grown in insect cells (C6/36) for use in this study. Here, viruses that were derived from SA_{14} and SA_{14}-14-2 viruses are designated SA(V) and SA(A), respectively, as described in Materials and Methods. The size of the plaques of these viruses displayed on BHK-21 monolayer cells were examined (Fig. 1). The result suggests that the plaque-size phenotypes did not change during plaque purification in BHK-21 cells and propagation in C6/36 cells. Furthermore, mouse neurovirulence tests on plaque-purified viruses were performed by intracerebrally injecting the viruses into suckling, 2-week-old and 4-week-old ddY mice to confirm that their neurovirulence phenotypes were also unchanged (Table 2). One PFU of virus SA(V) seemed to be enough for 4-week-old mice to die of

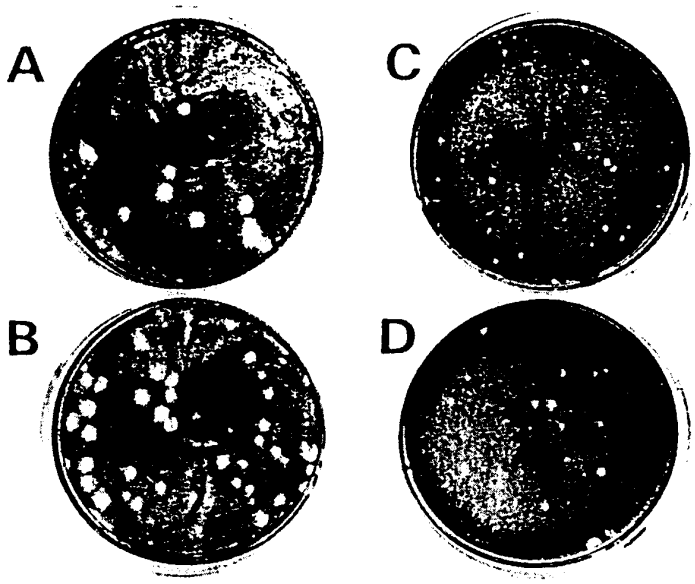

Fig. 1. Plaques displayed on monolayers of BHK-21 cells. The virulent SA_{14} virus and attenuated SA_{14}-14-2 were plaque purified in BHK-21 cells and propagated in C6/36 cells, as described in Materials and Methods. Viruses thus obtained were designated SA(V) and SA(A), respectively, in this study. Plaques of viruses SA_{14} (A), SA(V) (B), SA_{14}-14-2 (C), and SA(A) (D) displayed on BHK-21 cells were stained with crystal violet and are shown in this figure.

Table 2. Mouse neurovirulence tests with intracerebral route

Amount of virus (PFU)	SA(V)	SA(A)			
				Suckling	
	4 week old	4 week old	2 week old	1st exp.[c]	2nd exp.
10^7	0/10[a]	ND[b]	ND	ND	ND
10^6	0/10	10/10	10/10	0/31	0/16
10^5	0/10	ND	10/10	ND	0/8
10^4	0/10	ND	10/10	ND	0/8
10^3	0/10	ND	ND	ND	ND
10^2	0/10	ND	ND	ND	ND
10^1	0/10	ND	ND	ND	ND
10^0	5/10	ND	ND	ND	ND
10^{-1}	10/10	ND	ND	ND	ND

[a] Number of survival mice/number of mice inoculated with virus.
[b] Experiment was not done.
[c] Experiment.

encephalitis, while 2- or 4-week-old mice injected with 10^6 PFU of virus SA(A) did not develop any clinical symptoms, and only suckling mice were sensitive to virus SA(A) (Table 2). Thus, viruses SA(V) and SA(A) appear to have biological properties that are characteristic of viruses SA_{14} and SA_{14}-14-2, respectively.

Cloning and sequencing experiments

Double-stranded cDNAs were prepared from virion RNAs, as described in Materials and Methods, and digested with appropriate restriction enzymes selected by taking the previously determined nucleotide sequences of JEV genomes (6,7) into consideration. DNA fragments thus obtained are joined to cloning vector pUC18 or pUC19 and replicated in competent *E. coli*. Map positions of the JEV-cDNAs were determined by hybridization with specific DNA probes prepared from cDNAs of other JEV genomes (6,7) or by distribution of cleavage sites of restriction enzymes. Thus all cDNA clones selected were physically mapped and the main clones used for sequencing are shown in Fig. 2. Assembly of cDNA clones covers all of the genome except the short stretch at the 5′ terminal region.

Approximately 50 kinds of synthetic DNA primers were synthesized with each 200-nucleotide distance for sequencing cDNAs and virion RNAs. Primer exten-

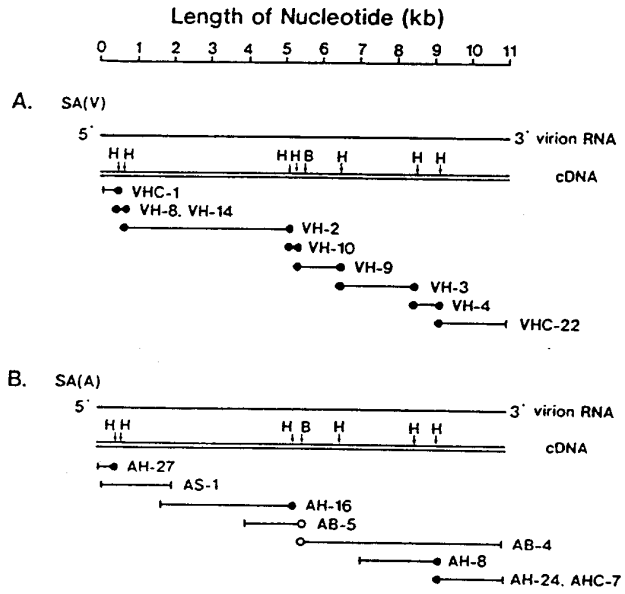

Fig. 2. (A) Restriction cleavage sites on SA(V) and (B) SA(A) cDNAs and the cDNA clones obtained. H and B represent restriction cleavage sites for *Hin*dIII and *Bam*H1, respectively. *Hin*dIII and *Bam*H1 cleavage sites used for molecular cloning are indicated by closed circles and open circles, respectively, at the ends of cDNA clones. Other ends of cDNAs appear to be connected with vector DNA by blunt-end ligations. The length of nucleotides from the corresponding 5′ terminus of the JEV genome is shown in kilobases at the top of the figure.

sion reactions were carried out to determine the nucleotide sequences of cDNAs and RNAs, as described in Materials and Methods. The primer extension method was also employed for sequencing the genome region close to the 5′ terminus, where sequence was not contained in the cloned cDNAs. Nucleotide sequences thus obtained were combined with the aid of reference nucleotide sequences of the genomes of JaOArS982 (6) and the Beijing-1 (7) strains. Thus, the total nucleotide sequences of both the SA(V) and SA(A) genomes were determined, except for two nucleotides at the 5′ terminus, which were difficult to identify by the primer extension method only (6,7), and 16 nucleotides at the 3′ terminus whose sequence was included in the synthetic oligonucleotide primer used for the reverse transcription. The nucleotide sequence of the SA(A) genome and the deduced amino acid sequence are shown in Fig. 3. A single ORF spanning 10,296 nucleotides encoding a polyprotein of 3432 amino acid residues is observed, flanked by 95 bases at the 5′ end and 585 bases at the 3′ end. The genome organization indicated in Fig. 3 follows those of the yellow fever virus (YFV) genome reported by Rice et al. (2) and Chambers et al. (10), and the Kunjin virus genome reported by Speight et al. (8,9).

Comparative sequence analysis

The nucleotide sequence of the attenuated strain SA(A) was compared with that of the parent SA(V) by computer analysis, and genetic variations that occurred during attenuation processes were identified. As a result, 57 nucleotide substitutions were found to disperse all over the genome (Table 3). Of these, 24 are amino-acid changes and the rest are silent mutations. As shown in Table 3, many missense mutations are located in viral envelope protein E, although every viral protein, except prM, contains missense mutation(s). Many base substitutions in the genome regions encoding NS2a, NS4b, and a putative viral replicase NS5 occurred in the third letter position of the in-phase codons (Table 3). This resulted in a low frequency of amino acid changes in these coding regions. This result may indicate that conservation of amino acid sequences of these portions of the nonstructural proteins is more important for JEV replication than conservation of those of the viral envelope protein.

Comparative sequence study was also performed on nucleotide sequences of four different JEV strains, JaOArS982, Beijing-1, SA(V), and SA(A). It should

Fig. 3. Nucleotide sequence of the genome of SA(A) and the predicted amino acid sequences of viral polyproteins. The RNA sequence deduced from the corresponding cDNA sequence is shown. The genome organization indicated here follows those of the YFV genome reported by Rice et al. (2) and Chambers et al. (10), and of the Kunjin virus genome by Speight et al. (8,9). Two bases at the 5′ terminus are not identified and follow the previously determined nucleotide sequence of JEV genome (6,7). The sequence of 16 nucleotides at the 3′ terminus also follows the previous data (6,7), and a synthetic oligonucleotide of this sequence was used as a primer for the synthesis of the cDNA, as described in Materials and Methods.

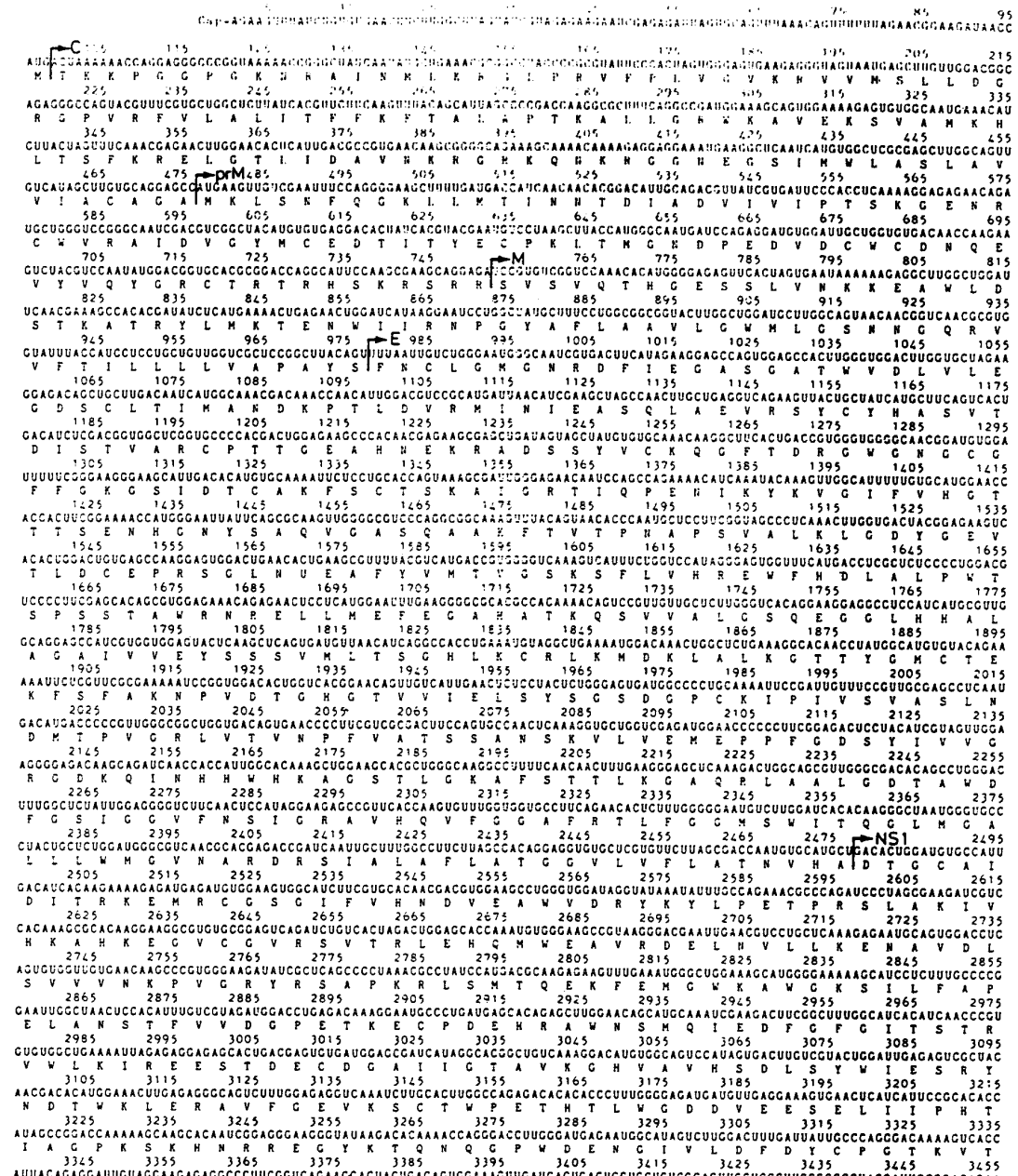

Fig. 3.

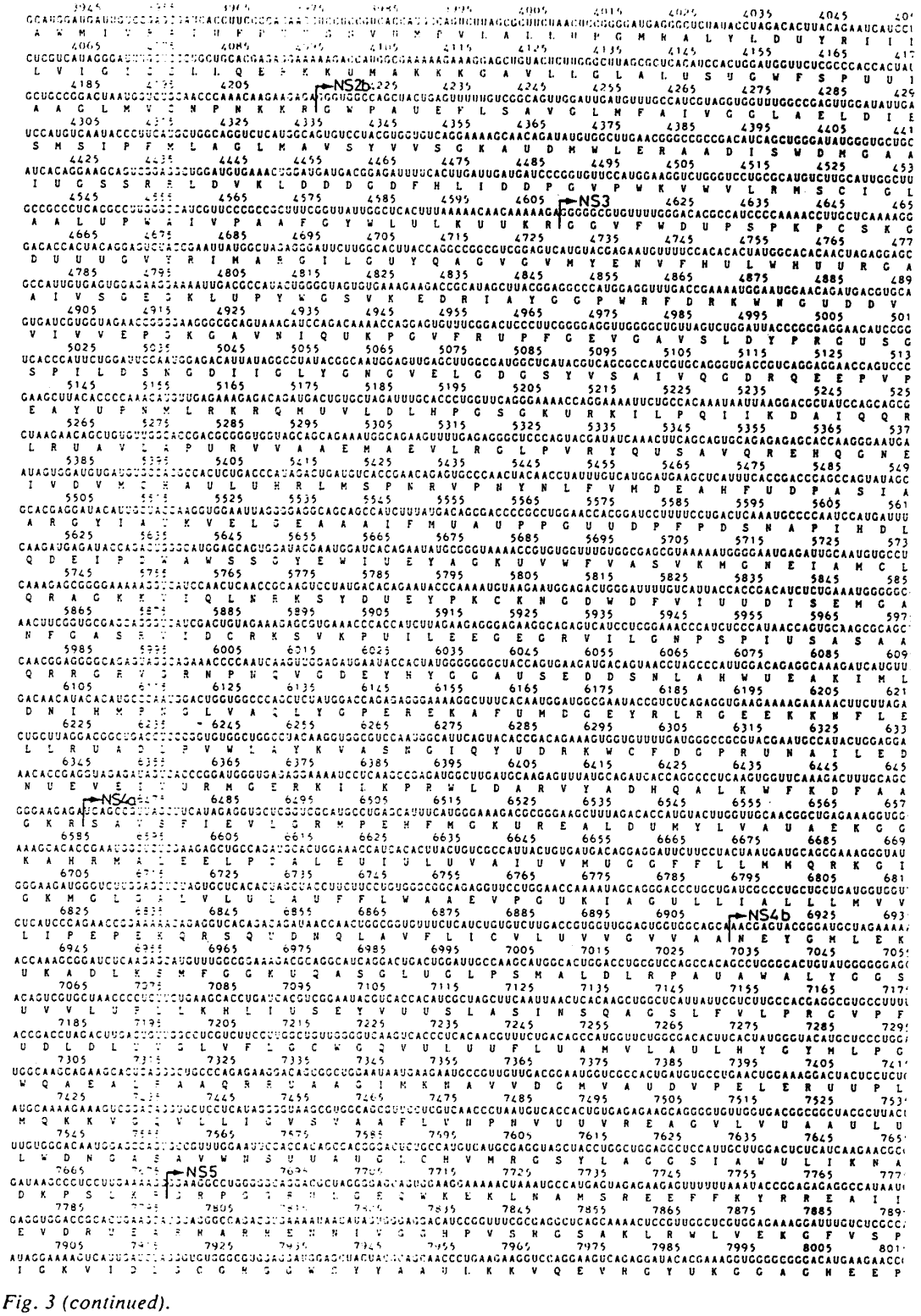

Fig. 3 (continued).

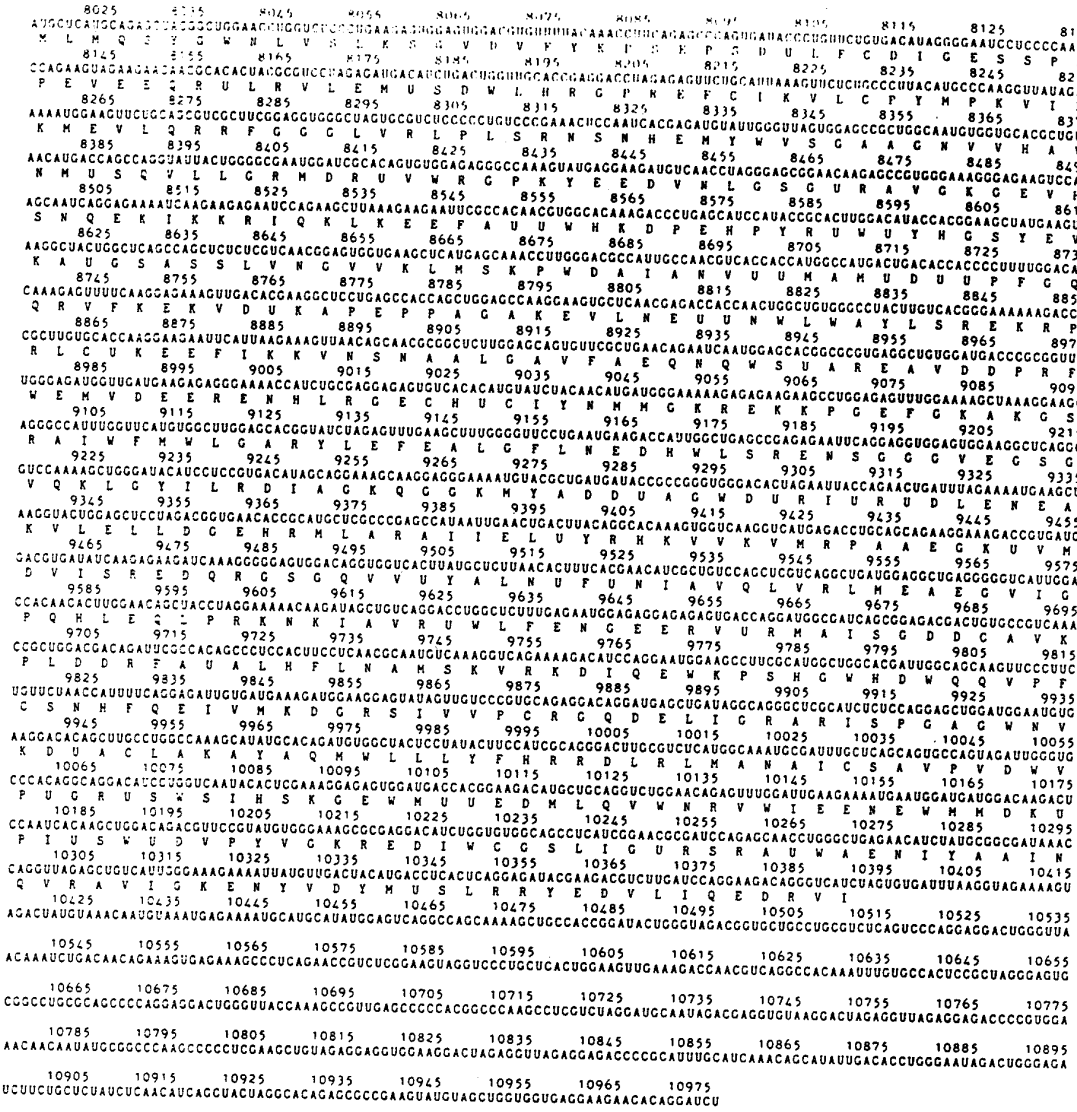

Fig. 3 (continued).

be noted that there are several wrong nucleotide sequences in a published genome sequence of Beijing-1 strain (7) because of the misreading of sequence ladders. The corrected nucleotide sequence of the Beijing-1 genome has already been sent to GenBank. As to the nucleotide sequence of the noncoding regions, highly homologous sequences are observed in both termini of the genome (Fig. 3 and refs. 6,7). Conservation of these nucleotide sequences must be very important for JEV replication, although the biological functions of these parts of the genome have not yet been elucidated. Differences in the primary structure of the coding

Table 3. Comparison of genome sequences between SA(V) and SA(A) strains

Substitutions					Substitutions				
Nucleotide position	Base		Amino acid		Nucleotide position	Base		Amino acid	
	SA(A)	SA(V)	SA(A)	SA(V)		SA(A)	SA(V)	SA(A)	SA(V)
39	A	T*[b]			4782 (1)	G	A	Val	Met*
292 (2)[a]	C	T	Ser	Leu*	4825 (2)	A	G	Lys	Arg
1061 (3)	C	T			4921 (2)	G	C	Gly	Ala*
1217 (3)	C	T			4922 (3)	C	T		
1296 (1)	T	C	Phe	Leu*	5634 (1)	T	A	Trp	Arg*
1389 (1)	A	G	Lys	Glu*	6008 (3)	T	C		
1503 (1)	G	A	Val	Ile*	6425 (3)	G	A		
1506 (1)	G	A	Ala	Thr*	6634 (2)	C	T	Thr	Ile*
1769 (3)	T	G	His	Gln*	6728 (3)	A	G		
1813 (2)	T	A	Met	Lys*	6944 (3)	G	A		
1921 (2)	T	C	Val	Ala*	7121 (3)	T	C		
2293 (2)	G	A	Arg	Lys*	7193 (3)	T	C		
2441 (3)	A	G			7227 (1)	G	A	Val	Ile*
2691 (1)	A	C			7655 (3)	G	T		
2843 (3)	C	T			7736 (3)	T	C		
3351 (1)	A	G	Ser	Gly*	8099 (3)	T	C		
3493 (2)	T	G	Met	Arg*	8276 (3)	T	C		
3528 (1)	C	G	His	Asp*	8394 (1)	T	C		
3539 (3)	A	T	Lys	Asn*	8832 (1)	T	C	Tyr	His*
3599 (3)	A	G			8882 (3)	T	A		
3652 (2)	T	C	Val	Ala*	8891 (3)	T	C		
3677 (3)	T	C			9688 (2)	C	T	Ala	Val*
3776 (3)	T	C			9695 (3)	A	G		
3801 (1)	T	C			9818 (3)	T	C		
3929 (3)	**T**	**C**			**10046 (3)**	**A**	**G**		
4106 (3)	**G**	**A**			**10139 (3)**	**T**	**C**		
4403 (3)	T	G	Asp	Glu*	10217 (3)	C	T		
4408 (2)	G	A	Gly	Asp*	10428	C	T*		
					10784	T	C		

Table 4. Genetic variation in the translated region among JEV strains

JEV	versus	JEV	Nucleotide substitutions (%)	Amino acid substitutions (%)	Missense mutations (%)
SA(A)		Beijing-1	2.75 (283)[a]	1.37 (47)[b]	16.6[c]
		JaOArS982	1.94 (200)	1.08 (37)	18.5
		SA(V)	0.52 (54)	0.07 (24)	44.4
SA(V)		Beijing-1	2.34 (241)	0.82 (28)	11.6
		JaOArS982	1.46 (150)	0.17 (18)	12.0
Beijing-1		JaOArS982	2.87 (296)	0.79 (27)	9.1

[a] Number of nucleotide substitutions observed is indicated in parenthesis.
[b] Number of amino acid substitutions observed is indicated in parenthesis.
[c] Ratio of missense mutations to the total nucleotide substitutions.

sequences among these four JEV strains were determined with the aid of a computer (Table 4). The data suggest that three virulent JEV strains that were independently isolated are genetically related to each other. The ratios of missense mutations to the total nucleotide substitutions between any two JEV strains were calculated and are shown in Table 4. Interestingly, the highest rate (44.4%) of missense mutations among those between any two strains of genomes was observed when the SA(V) and SA(A) genomes were compared with each other, although the genetic variation between these two strains was the smallest. This observation may reflect mutations occurring in the attenuation processes shown in Table 1, during which the wild JEV strain is adapted to PHK cells. If this is the case, there must be a certain selection pressure on JEV replication in PHK cells, leading to adaptation mutations, some of which may contribute to the attenuation phenotype of JEV.

Amino acids in viral proteins of the virulent JaOArS982 (6) and Beijing-1 (7) strains that correspond to amino acid differences between SA(V) and SA(A) were examined. Common amino acids among the three virulent strains JaOArS982, Beijing-1, and SA(V) are indicated by asterisks in Table 3. It is of interest that all the amino acids, except for one in NS3, are common in these virulent strains. Furthermore, out of three point mutations in the untranslated regions, two nucleotides of the SA(V) genome, indicated by asterisks in Table 3, are common in the genomes of other virulent strains. Thus many mutations may be determinants of the attenuation phenotype.

Discussion

Recently, Eckels et al. (20) proposed a vaccine lot of strain SA_{14}-14-2 that was adapted to an alternative cell substrate, primary canine kidney (PCK) cell cultures. Quality control of the attenuated vaccine may be easier in PCK cell cultures as compared with PHK cell cultures (20). Although the live, attenuated JE strain

appears to be fairly stable in their attenuation phenotype during the replication in PHK and PCK cells, the molecular genetic background of the attenuation phenotype is totally unknown at present. Elucidation of the genetic background may be essential to use the live attenuated vaccine for humans worldwide, since a similar study has already been conducted with live attenuated poliovirus vaccines (16,21,22) and the results have been used effectively to test the genetic stability of the vaccine (S. Abe, N. Iizuka, K. Tago, and A. Nomoto, manuscript in preparation) and to construct new vaccine candidates (21,23,24).

Both attenuated SA_{14}-14-2 and virulent virus SA_{14} strains are closely related to each other genetically, with only 57 nucleotide differences in the entire 10,976-nucleotide long genome. Furthermore, several biological markers that may correlate with the virulence of the JEV have been well investigated. These markers include mouse neurovirulence and plaque size in LLC-MK2, CE, and BHK-21 cells, which can easily be tested in the laboratory. Thus these two JEV strains provide an excellent experimental model to investigate the molecular mechanisms of JEV attenuation.

There must be many kinds of mutations that lead to viral attenuation. In the case of all three poliovirus vaccine strains, strong determinants of the attenuation phenotype were discovered to reside in the 5′ untranslated region, and it is therefore possible that the attenuation phenotype may be due to lowered efficiency in a certain step of the viral replication in the central nervous system (16,21,22,24). On the other hand, Lustig et al. (25) reported that mutation(s) reducing the neurovirulence of Sindbis virus exists in the genome region encoding the surface glycoproteins. Therefore, a decrease in the affinity of the virus to cellular receptors may be involved in the attenuation of Sindbis virus. In this study, we identified one and two point mutations in the 5′ and 3′ untranslated sequences, respectively. Furthermore, eight amino acid replacements were identified in the E protein of JEV that corresponded to the surface glycoprotein of Sindbis virus. For the identification of mutations that influence the attenuation phenotype, it is essential to construct chimera viruses between the two strains. Infectious cDNA clones of both strains are currently under construction. This kind of study should also elucidate the relationship between mutations for the adaptation to PHK cells and those for attenuation in mouse neurovirulence.

Acknowledgments

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Nucleotide Sequence of the Virulent SA-14 Strain of Japanese Encephalitis Virus and Its Attenuated Vaccine Derivative, SA-14-14-2

SORACHAI NITAYAPHAN,[1] JOYCE A. GRANT, GWONG-JEN J. CHANG, AND DENNIS W. TRENT[2]

Division of Vector-Borne Infectious Diseases, Center for Infectious Diseases, Centers for Disease Control, Public Health Service, U.S. Department of Health and Human Services, Post Office Box 2087, Fort Collins, Colorado 80522

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The attenuated SA-14-14-2 strain of Japanese encephalitis (JE) virus has been used to immunize people in the People's Republic of China. Oligonucleotide fingerprints of the parent SA-14 and vaccine strain indicate that multiple genetic changes occurred during attenuation of the virus. We have cloned and sequenced the genomes of both the virulent SA-14 and attenuated SA-14-14-2 viruses to define molecular differences in the genomes. Forty-five nucleotide differences, resulting in 15 amino acid substitutions, were found by comparing sequences of the SA-14 and SA-14-14-2 genomes. Transversion of U to A occurred at position 39 in the 5′-noncoding region of SA-14-14-2 and another SA-14 vaccine derivative SA-14-5-3. A single nucleotide change in the capsid gene of SA-14-14-2 altered a single amino acid which changed its predicted secondary structure. A silent nucleotide change was found in the prM gene sequence and the M-protein was unchanged. There are seven nucleotide differences, resulting in five amino acid changes, in the E glycoprotein sequence of the two viruses. Nine amino acid differences were found in the nonstructural proteins of SA-14 and SA-14-14-2: one in NS2A, two in NS2B, three in NS3, one in ns4a, and two in NS5. A single nucleotide change at position 10,428 in the 3′-noncoding region is vaccine virus-specific. The nucleotide and deduced amino acid sequences of the vaccine strain SA-14-14-2, the parent virus SA-14, and virulent strains JaOArS982 and Beijing-1 have been compared and are highly conserved. © 1990 Academic Press, Inc.

INTRODUCTION

Japanese encephalitis (JE) virus, a mosquito-transmitted flavivirus of veterinary importance, produces central nervous system disease in humans (Buescher and Scherer, 1959). Epidemics of JE are a major cause of morbidity and mortality throughout temperate areas of Asia and the northern part of tropical Southeast Asia, including Thailand (Gunakasem *et al.*, 1981). Epidemics of 2000 cases and 400 deaths occur annually in the northern provinces of Thailand (Hoke *et al.*, 1988). For this reason the World Health Organization has targeted JE virus for vaccine development (Brandt, 1988).

The live-attenuated JE virus vaccine strain SA-14-5-3 was developed in the People's Republic of China from the neurovirulent SA-14 strain by passage in primary hamster kidney cells (Yu *et al.*, 1962; Eckels *et al.*, 1988). This attenuated vaccine has been used to immunize approximately 5 million children in the People's Republic of China (Yu *et al.*, 1973). The SA-14-5-3 vaccine virus is genetically unstable and overattenuated (Huang, 1982). The attenuated JE vaccine strain SA-14-14-2 was developed by passaging the SA-14-5-3 virus subcutaneously five times in infant mice followed by plaque purification. The resulting SA-14-14-2 strain used to immunize thousands of children in China is more stable and immunogenic than the parent SA-14-5-3 virus (Yu *et al.*, 1988). The SA-14-14-2 virus did not become virulent for mice after passage in mosquitoes or cell cultures (Hori *et al.*, 1986a,b; Eckels *et al.*, 1988). Eckels *et al.* (1988) adapted this virus to grow in primary canine kidney (PCK) cells and observed that it produced small plaques in LLC-MK2 cells and was not temperature-sensitive.

The JE virus RNA genome is approximately 11-kb long, single-stranded, positive-sense in polarity, capped at the 5′-terminus, and lacks a poly(A) tract at the 3′-terminus (McAda *et al.*, 1987; Takegami *et al.*, 1986; Sumiyoshi *et al.*, 1987; Hashimoto *et al.*, 1988). Short noncoding regions are located at both the 3′- and the 5′-terminus. Flavivirus particles contain three structural proteins translated from the 5′ one-quarter of the genome in the order: nucleocapsid protein (C; 13,500 kDa), a nonglycosylated envelope protein (M; 8700 kDa), and the major envelope protein (E; 53,000 kDa) which may or may not be glycosylated (see Westaway, 1987, for review). Nonstructural proteins NS1 to NS5 are translated from the coding region immediately following the E-protein.

To understand the molecular basis of flavivirus virulence and attenuation we have cloned and sequenced genomes of the virulent parent SA-14 virus and its at-

[1] Present address: Armed Force Research Institute of the Medical Sciences, 315/6 Rajvithi Road Bangkok 10400, Thailand.

[2] To whom requests for reprints should be addressed.

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tenuated derivative SA-14-14-2. We report the nucleotide and deduced amino acid sequences of these two viruses and compare them with the sequences of virulent JE strains JaOArS982 (Sumiyoshi *et al.*, 1987) and Beijing-1 (Hashimoto *et al.*, 1988).

MATERIALS AND METHODS

Virus strains

SA-14 (PCK-3) and SA-14-14-2 (PCK-7) viruses were obtained from Dr. Kenneth H. Eckels, Department of the Army, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D.C. The viruses were isolated and selected in the People's Republic of China by Dr. Li Ho-Ming and Dr. Yu Yong Xin at the National Institute for the Control of Pharmaceutical and Biological Products, Beijing. The SA-14-5-3 strain, attenuated parent of SA-14-14-2, was obtained from the San Juan Laboratories, Division of Vector-Borne Infectious Diseases, Centers for Disease Control, San Juan, Puerto Rico. Virulence of the SA-14 strain and attenuation of the SA-14-14-2 and SA-14-5-3 strains was confirmed prior to molecular cloning.

Viruses were propagated in C6/36 *Aedes albopictus* cells infected at a multiplicity of 0.05 to 0.1 and the supernatant fluid was harvested after 5 days of incubation at 28°. Virus was purified and the RNA extracted as previously described (Trent *et al.*, 1981).

Molecular cloning of SA-14 and SA-14-14-2 viral RNA

Five oligonucleotide primers complementary to the published sequence of strain JaOArS982 were used to prime first strand cDNA synthesis on the RNA genome template (Sumiyoshi *et al.*, 1987; Takegami *et al.*, 1986). The primers were located on the genome at the following nucleotide positions: primer 1, 10,940 to the 3'-end; primer 2, 10,818 to 10,841; primer 3, 4351 to 4373; primer 4, 1999 to 2027; and primer 5, 6341 to 6362. These oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer (Applied Biosystems, Foster City, CA)[3] and purified by polyacrylamide gel electrophoresis. First strand cDNA synthesis was also primed using degraded calf thymus DNA (Trent *et al.*, 1987). The cDNA was synthesized from JE virus RNA by the method of Gubler and Hoffman (1983) as modified by Kinney *et al.* (1986).

Poly(dC)-tailed cDNA was annealed to poly(dG)-tailed bluescript (SK; M13+; Stratagene) or poly(dG)-tailed pUC18 plasmid at the *Pst*I site. *Escherichia coli* strain DH5 α (Bethesda Research Laboratories), a derivative of strain DH5 (Raleigh *et al.*, 1989), was made competent by sequential washes with cold (4°) magnesium chloride and calcium chloride (100 m*M* each), followed by resuspension in one-tenth of the original culture volume of 50 m*M* calcium chloride. Ampicillin-resistant white colonies were grown overnight in 2X-YT broth, and plasmids were extracted (Holmes and Quigley, 1981) and screened for size by agarose gel electrophoresis. Recombinant plasmids containing cDNA inserts greater than 2000 bp were screened by dot-blot hybridization using ^{32}P-end-labeled oligonucleotide primers (Grunstein and Hogness, 1975).

JE virus cDNA to be sequenced was subcloned into M13-mp18 or M13-mp19 (Yanisch-Perron *et al.*, 1985) and sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977, 1980). Overlapping clones were prepared to facilitate sequencing of large inserts (Dale *et al.*, 1985). Primer-directed RNA sequencing was performed by the method of Biggin *et al.* (1983). Double-strand DNA sequencing was carried out by the methods of Zagursky *et al.* (1985) and Bartlett *et al.* (1986).

Computer analysis of nucleic acid sequence data was accomplished with DNASIS and PROSIS software (Hitachi of America, San Bruno, CA). Cleavage sites in the putative polyprotein were assigned as described by Rice *et al.* (1985) and Speight *et al.* (1988).

RESULTS

Cloning of SA-14-14-2 and SA-14

Nucleotide sequences of SA-14-14-2 and SA-14 virus genomes were determined from cDNA clones illustrated in Fig. 1. Restriction sites used to align the cDNA clones are indicated at the top of this figure. Clone CT-5 was obtained by nonspecific priming of first strand synthesis using oligonucleotides prepared from degraded calf-thymus DNA (Fig. 1). Clone Ps-72 was obtained using primer 2. Clones P11-66, P11-63, V11-3, and V11-61 were synthesized using primer 1; clones Pa-7/9, Pa-7/33, and pSN7-124 were cloned using primer 4; clone pVa121, using primer 3; and clone V6/28, using primer 5. Sequence analysis indicated that some of the oligonucleotide primers complementary to one region initiated synthesis elsewhere in the genome in addition to the specific site (Despres *et al.*, 1987; R. Kinney, personal communication).

Molecular structure and cloning of SA-14-14-2 and SA-14 viral RNA

We have determined the nucleotide sequence of the coding regions, 5'-noncoding region, and 539 nucleo-

[3] Use of source is for identification only and does not constitute endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

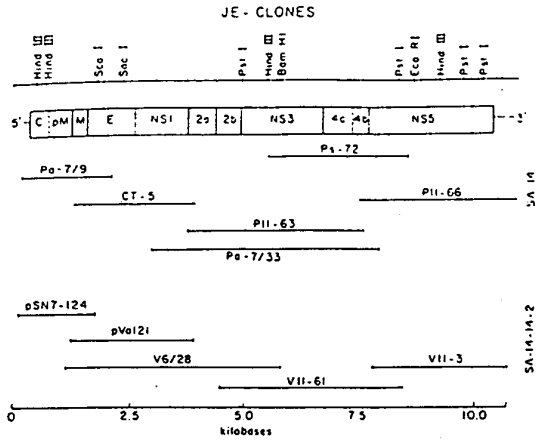

FIG. 1. Strategy for obtaining the complete nucleotide sequences of Japanese encephalitis SA-14 and SA-14-14-2 genomic RNAs. cDNA was sequenced in overlapping clones, as determined by restriction mapping and nucleotide sequencing of both ends of the clones. Clones Pa7/9, CT-5, P11-63, Pa7/33, Ps-72, and P11-66 were derived from the genome of virus strain SA-14; clones pSN7-124, pVa121, V6/28, V11-61, and V11-3, from the genome of SA-14-14-2.

tides in the 3'-noncoding region of both SA-14-14-2 and the SA-14 JE virus genomes. Both strands of the cDNA clones of both JE viruses examined in this study have been completely sequenced. Nucleotide sequences of the extreme 3'-termini of the RNAs were not present in any of the clones and could not be determined by direct RNA sequencing using primer 1 derived from the 3'-terminal JE virus genome sequence (Sumiyoshi *et al.*, 1987). The nucleotide sequence of the 5'-noncoding region was determined by direct sequencing of the virus RNA using a specific primer complementary to the genome sequence at positions 97 to 121. The sequenced regions of the genomes of SA-14 and SA-14-14-2 JE viruses were 10,935 nucleotides long and the genes organized as previously described for JaOArS982 and Beijing-1 strains of JE virus (Sumiyoshi *et al.*, 1987; Hashimoto *et al.*, 1988). Glycosylation sites assigned by the sequence ASN-X-Ser/Thr were identical for SA-14, SA-14-14-2, and the other sequenced JE virus strains. Both SA-14-14-2 and SA-14 virus RNAs contained a 95 nucleotide 5'-terminal nontranslated region (Fig. 2).

Nucleotide and deduced amino acid sequence comparison of viral RNAs

Comparison of the nucleotide and deduced amino acid sequences of the virulent parent virus SA-14 with the attenuated vaccine strain SA-14-14-2 revealed 45 nucleotide sequence differences. In the noncoding region of the genome there was a U to A transversion at position 39 and a U to C transition at position 10,428 in the sequence. Nucleotide sequence changes in the coding region resulted in 15 amino acid substitutions: 1 in the capsid, 5 in the envelope, 1 in NS2A, 2 in NS2B, 3 in NS3, 1 in ns4a, and 2 in NS5 (Tables 1 and 2, Fig. 3). In the capsid region of SA-14-14-2 a nucleotide transition from U to C at nucleotide position 292 resulted in a nonconservative Leu to Ser substitution at position 65 in the capsid protein. This change altered the predicted secondary structure of the capsid protein (Chou and Fasman, 1978a,b,c; Rose, 1978). A single silent nucleotide substitution was detected in the prM region of the SA-14-14-2 genome. There were no nucleotide or amino acid alterations in the nucleotide sequence that encoded the mature M protein.

Seven nucleotide changes, resulting in five amino acid substitutions, were detected in the E glycoprotein gene of the vaccine strain SA-14-14-2 (Tables 1 and 2, Fig. 4). The Leu-to-Phe amino acid substitution at position E-107 is located in a highly conserved region of the flavivirus E protein (Lobigs *et al.*, 1987). Glu–Lys, Ile–Val, Glu–Lys, and Lys–Met substitutions occurred at envelope amino acid positions 138, 176, 243, and 279, respectively.

Of the 45 nucleotide differences detected in the genome of SA-14-14-2 virus, 34 changes were present in the nonstructural genes (Table 1). The SA-14-14-2 gene encoding NS1 contained a single silent nucleotide substitution. Nucleotide changes in the sequence of NS2A resulted in an Ile to Val amino acid substitution at position 46. Two nucleotide changes in NS2B resulted in Glu–Asp and Asp–Gly substitutions at amino acid positions 63 and 65, respectively.

Ten nucleotide differences, resulting in three amino acid substitutions (NS3-59, Met–Val; NS3-73, Arg–Lys; and NS3-105, Ala–Gly), were detected in protein NS3. There was an Ile–Val change in ns4a at amino acid position 225 and three additional silent mutations. There were 15 nucleotide changes in NS5, resulting in two amino acid substitutions: Glu to Lys at NS5-328 and His to Try at position NS5-386.

Comparison of SA-14-14-2 with virulent strains SA-14, JaOArS982, and Beijing-1

Comparison of the deduced amino acid sequences of SA-14 with the sequences of two other neurovirulent JE strains, JaOArS982 and Beijing-1 (Figs. 3 and 4), revealed a 97% conservation in the amino acid sequence. The deduced amino acid sequence of the SA-14-14-2 vaccine virus in comparison to the sequence of the three virulent JE strains contained 11 unique amino acid changes: 1 in the capsid, 5 in the E protein,

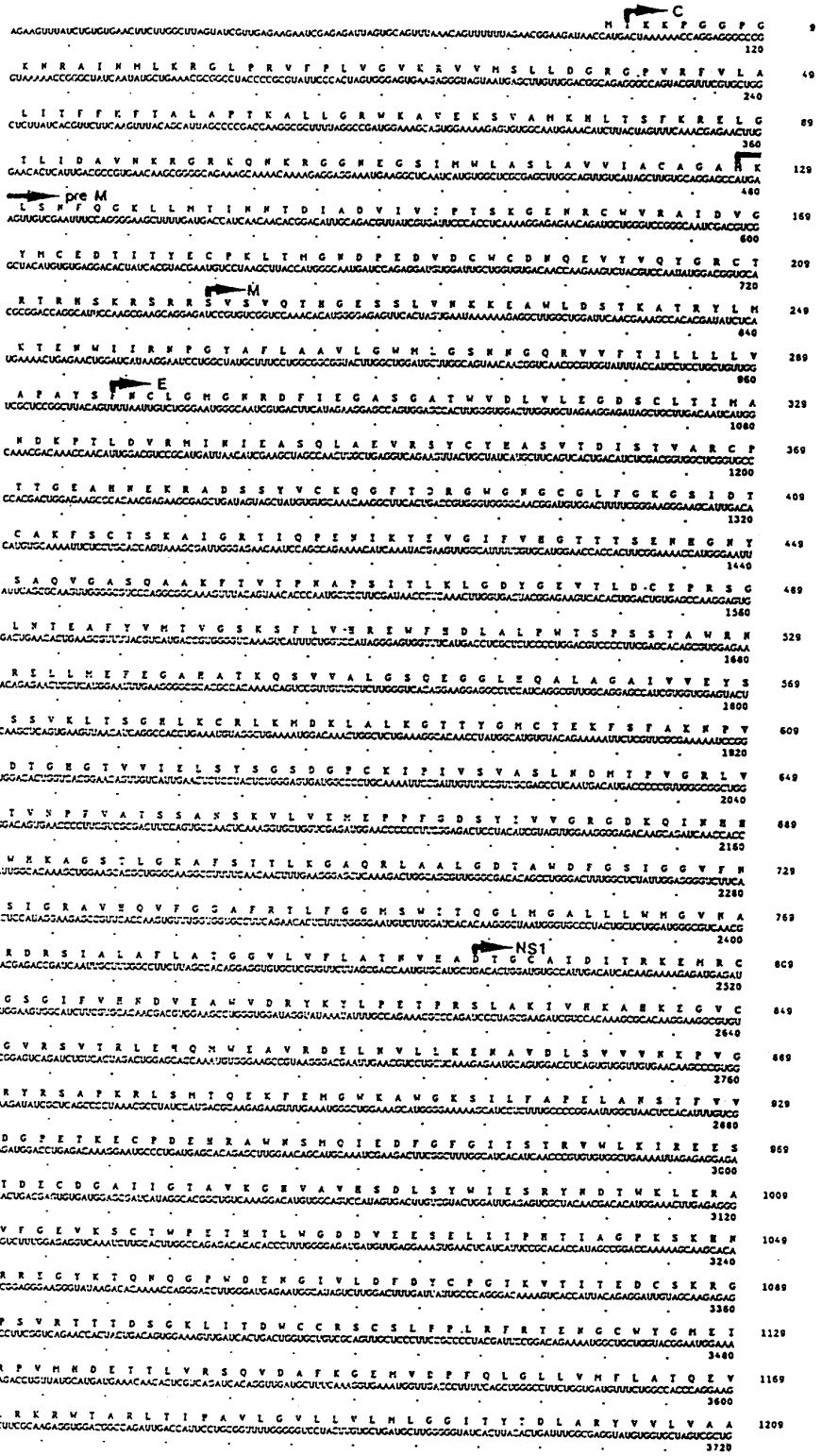

FIG. 2. Nucleotide sequence of the entire SA-14 RNA and the deduced amino acid sequence of the encoded polypeptides.

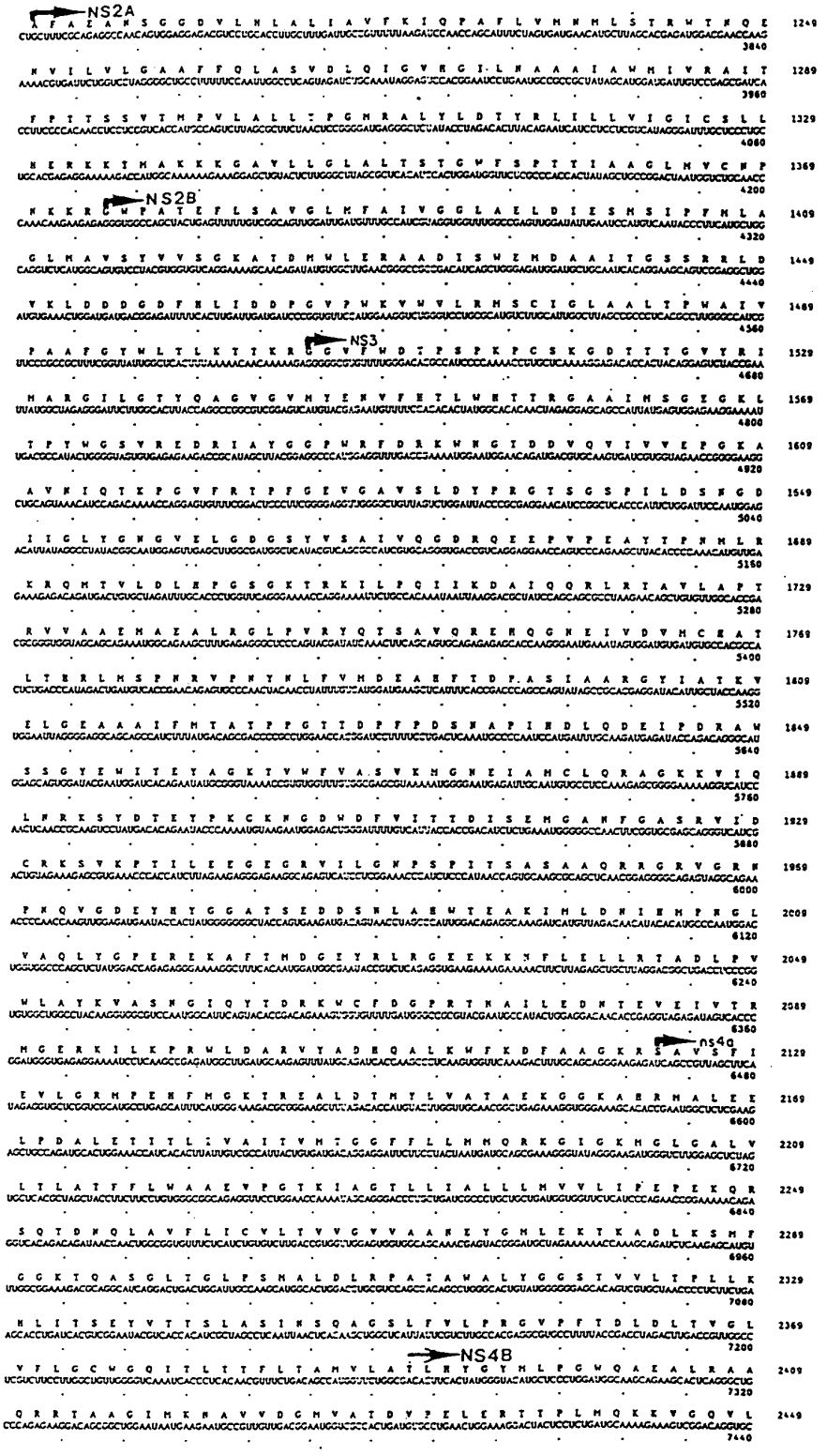

FIG. 2—*Continued*

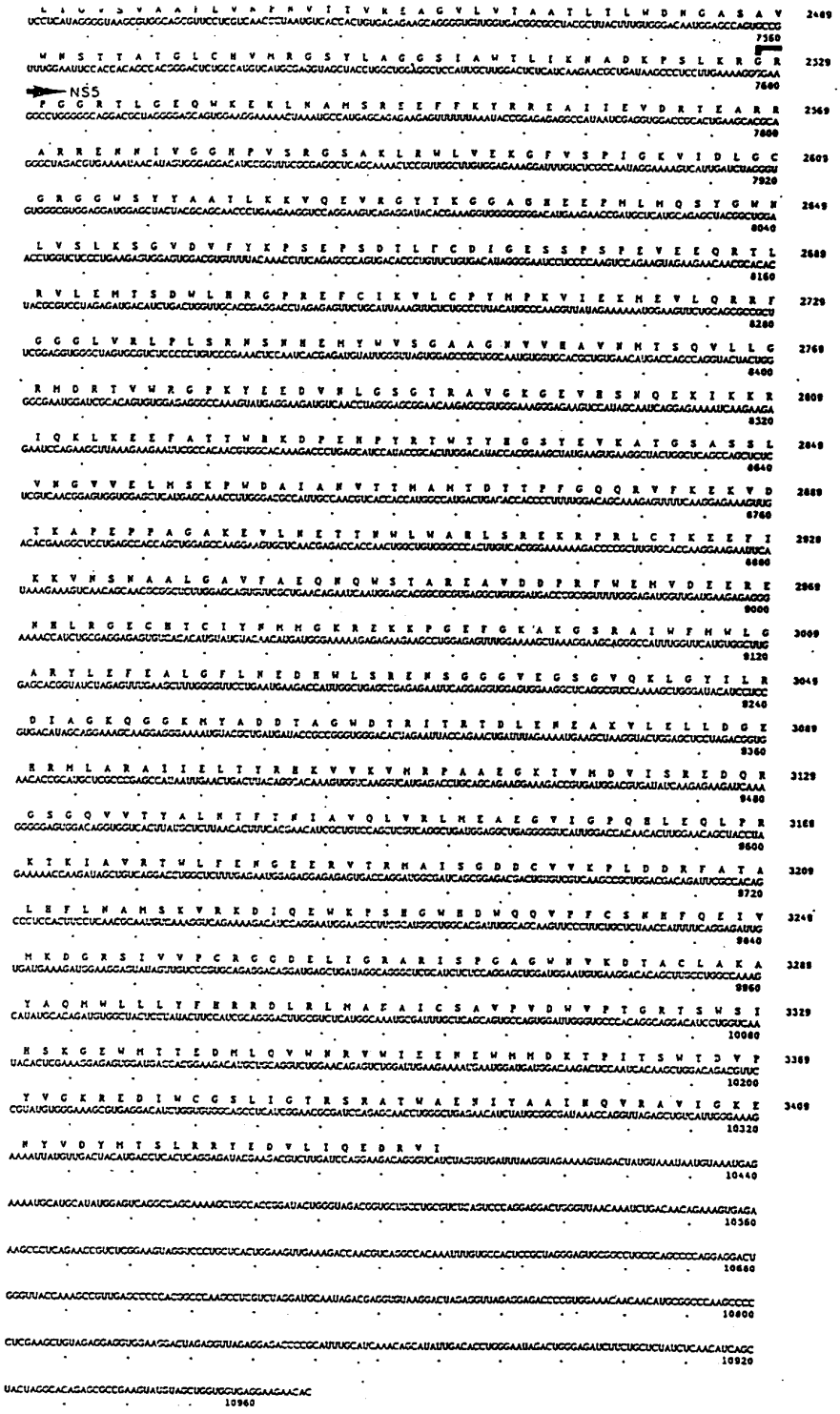

FIG. 2—*Continued*

TABLE 1

A SUMMARY OF DIFFERENCES BETWEEN THE SA-14 PARENT AND SA-14-14-12 VACCINE JE VIRUS STRAINS

Position		NT[a]		AA[b]		Position		NT		AA	
NT	AA	P	V	P	V	NT	AA	P	V	P	V
						6,425	NS3	A	G	—	
39	5'NC	U	A	Noncoding		6,728	ns4a	U	A	—	
292	C-65	U	C	Leu	Ser	7,121	ns4a	C	U	—	
578	prM	C	U	—		7,193	ns4a	C	U	—	
989	E	G	U	—		7,227	ns4a-225	A	G	Ile	Val
1061	E	U	C	—		7,736	NS5	C	U	—	
1296	E-107	C	U	Leu	Phe	7,805	NS5	U	C	—	
1389	E-138	G	A	Glu	Lys	7,871	NS5	U	C	—	
1503	E-176	A	G	Ile	Val	8,099	NS5	C	U	—	
1704	E-243	G	A	Glu	Lys	8,276	NS5	C	U	—	
1813	E-279	A	U	Lys	Met	8,394	NS5	C	U	—	
3662	NS1	G	U	—		8,658	NS5-328	G	A	Glu	Lys
3776	ns2a	C	U	—		8,832	NS5-386	C	U	His	Tyr
3801	ns2a	C	U	—		8,882	NS5	A	U	—	
3849	NS2a-46	A	G	Ile	Val	8,891	NS5	C	U	—	
4403	NS2b-63	G	U	Glu	Asp	9,695	NS5	G	A	—	
4408	NS2b-65	A	G	Asp	Gly	9,818	NS5	C	U	—	
4782	NS3-59	A	G	Met	Val	10,046	NS5	G	A	—	
4825	NS3-73	G	A	Arg	Lys	10,139	NS5	C	U	—	
4921-2	NS3-105	CU	GC	Ala	Gly	10,217	NS5	U	C	—	
5120	NS3	A	G			10,428	3'-NC	U	C	—	
5144	NS3	C	U	—							
5204	NS3	G	A	—							
6008	NS3	C	U	—							

[a] Nucleotide.
[b] Amino acid.

1 in NS2B, 2 in NS3, 1 in ns4a, and 1 in NS5 (Fig. 4). Of the 45 nucleotide differences between SA-14-14-2 and SA-14 virus strains, 38 were unique in the vaccine virus. An adenine substitution at position 39 in the 5'-noncoding region and the cytidine substitution at position 10,428 in the 3'-noncoding region of the SA-14-14-2 and SA-14-5-3 attenuated viruses were unique.

DISCUSSION

Molecular cloning and nucleotide sequence analysis of the genomes of the virulent SA-14 and attenuated vaccine SA-14-14-2 strains of JE virus have revealed 45 nucleotide differences in the sequences of the two viruses (Table 1). Sequence of the 41 3'-terminal nucle-

TABLE 2

AMINO ACID COMPARISONS OF THE E GLYCOPROTEIN OF JAPANESE ENCEPHALITIS STRAINS SA-14 AND SA-14-14-2 AND OF OTHER FLAVIVIRUSES

	Amino acid residue[b]								
Amino acid number[a]	SA-14	SA-14-14-2	WN	SLE	MVE	YF	DEN2	DEN4	TBV
107	L	F	L	L	L	L	L	L	L
138	E	K	E	E	E	V	T	T	T
176	I	V	Y	F	I	Q	T	V	S
243	E	K	E	E	E	E	K	K	E
279	K	M	K	T	K	K	L	H	H

[a] Amino acid position from amino terminal of E glycoprotein for both JE strains.
[b] Residue in the corresponding position.

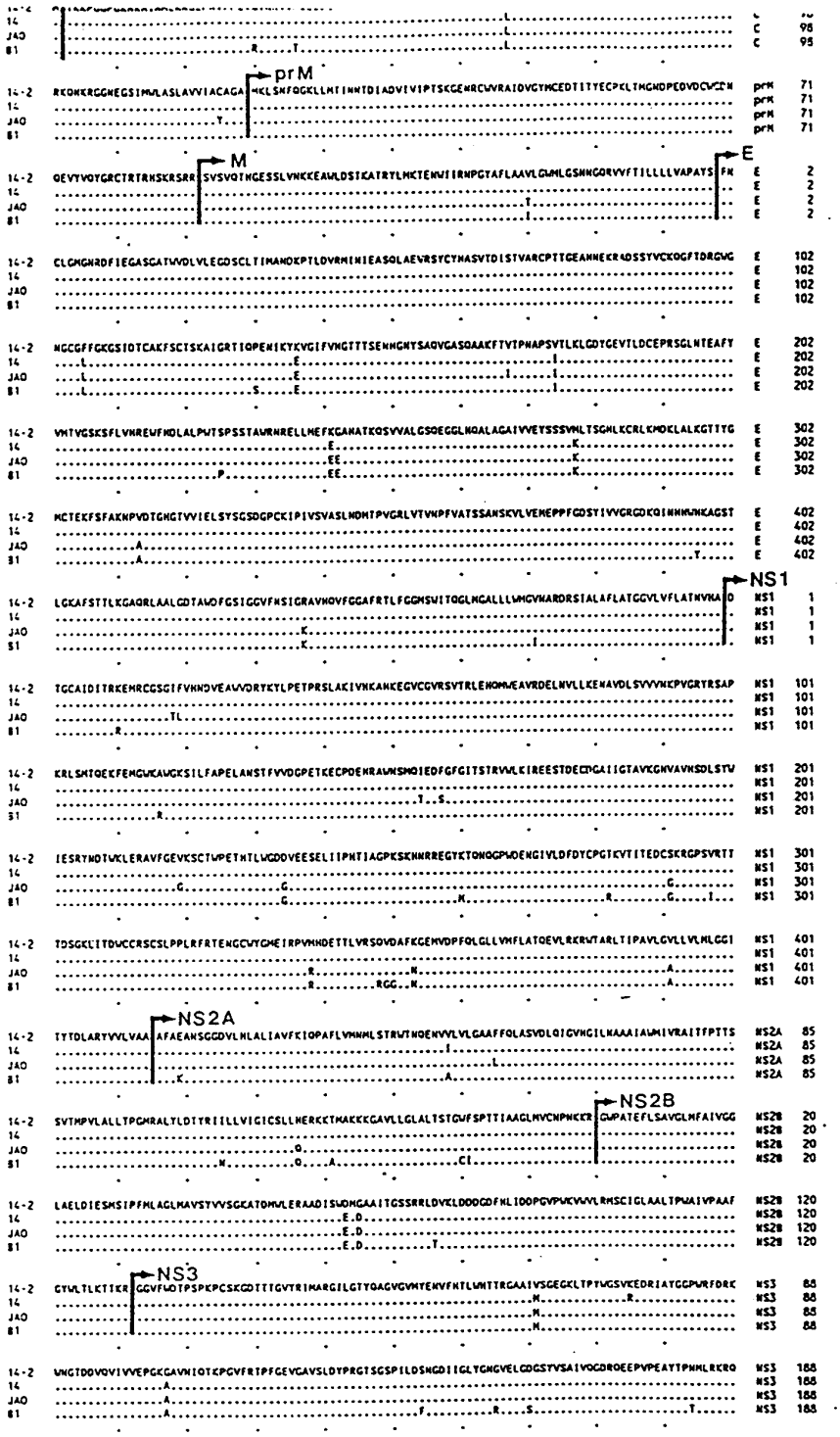

FIG. 3. Comparison of the deduced amino acid sequences of the structural protein (C, M, E) and nonstructural protein (NS1, ns2a, ns2b, NS3, ns4a, ns4b, and NS5) of SA-14 with those of SA-14-14-2, JaOArS982, and Beijing-1 strains. Deletions in the amino sequence of the Beijing-1 strain which were introduced to optimize the alignment, are indicated by a dash (-) in the sequence.

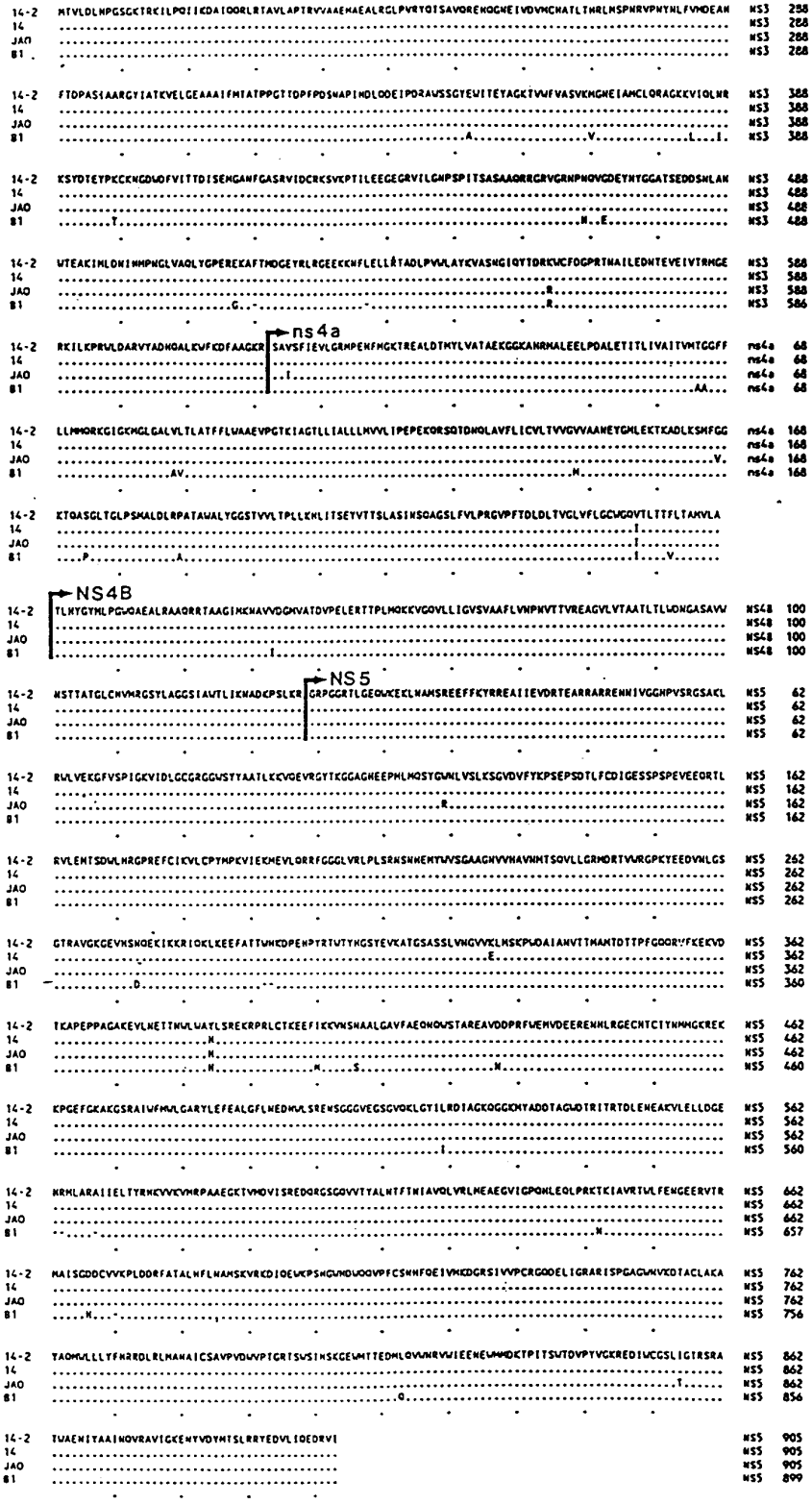

FIG. 3—*Continued*

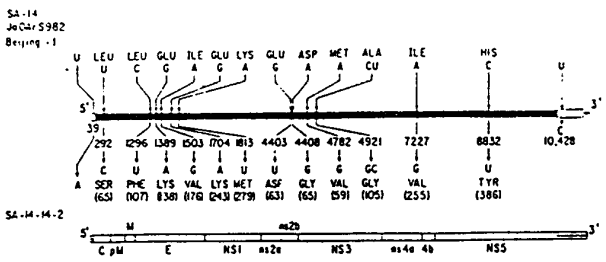

FIG. 4. Diagrammatic representation of nucleotide and predicted amino acid sequence differences between vaccine and virulent strains of Japanese encephalitis virus (the vaccine strain is SA-14-14-2; and the virulent strains are SA-14, JaOArS982, and Beijing-1).

otides of the genomes were not obtained and therefore could not be analyzed (Takegami *et al.*, 1986). Single nucleotide substitutions in the 5'- and 3'-noncoding regions of the genome altered the predicted "frying pan with a clover leaf handle" structure. Hashimoto *et al.* (1988) suggested this unique structure in translation of the virus polyprotein during the early stages of virus replication. Sequence changes within the coding region of the SA-14 genome resulted in 15 amino acid substitutions in the attenuated virus proteins (Table 1). The Leu to Ser substitution in the capsid protein of SA-14 could affect the predicted secondary structure of the protein and the interaction of the capsid protein with genomic RNA. Five amino acid differences in the E glycoprotein of the SA-14 and SA-14-14-2 viruses were noted. These changes in the E glycoprotein were primarily in hydrophilic regions within the carboxyl terminal half of the molecule. Changes in amino acids at positions 138, 243, and 279 in the E protein modified a trypsin cleavage site located in an immunologically important domain (Roehrig *et al.*, 1989). The significance of these alterations in relation to neurovirulence is unknown. Amino acid changes in the envelope protein at position 107 of the attenuated SA-14-14-2 virus occurred in flavivirus highly conserved region. This suggested that some biological function(s) of this conserved envelope domain may have been altered during attenuation (Table 2). Although there were no sequence changes in NS1, processing of SA-14-14-2 NS1 protein could be affected by the Glu to Leu change in the NS2A protein (Falgout *et al.*, 1989). Amino acid sequences of nonstructural proteins NS3 and NS5, thought to have protease and replicase functions, respectively, are highly conserved among the flaviviruses (Deubel *et al.*, 1988). Amino acid substitutions occurred in both NS3 and NS5 proteins of SA-14-14-2 virus (Table 1). These changes could affect viral RNA synthesis as well as processing of the polyprotein precursor.

Genetic changes responsible for flavivirus attenuation are not well understood at the molecular level although passage of the viruses in cell cultures may result in loss of virulence (Theiler and Smith, 1937; Yu *et al.*, 1962; Eckels *et al.*, 1980; Halstead *et al.*, 1984). Sequence analysis of parent/attenuated vaccine yellow fever virus was done to elucidate genetic changes associated with attenuation as a result of cell culture passage (Theiler and Smith, 1937). This study reveals multiple nucleotide differences between the parent and vaccine viruses throughout the genome (Hahn *et al.*, 1987). Many of the nucleotide changes resulting in amino acid substitutions in both yellow fever and JE virus are located in the envelope protein genes (Table 2). The envelope glycoprotein determines virus biological functions such as tissue tropism, cell fusion/infection, virus maturation, and induction of protective immunity (Westaway, 1987). Changes in the amino acid sequence of the envelope proteins of yellow fever and JE viruses are not similar in location or type of substitution. This suggests that either other genes are involved in determining virulence of the viruses or that the mechanism(s) of attenuation for the two viruses is different. Analysis of Murray Valley encephalitis (MVE) virus E protein sequences from viruses of different mouse virulence indicated there is no association between fatal cases and MVE E protein sequence (Lobigs *et al.*, 1988). Sequence analysis of dengue virus envelope protein from strains isolated from mild and severe cases of disease reveals no unique amino acid changes associated with disease severity (Blok *et al.*, 1989). The severity of disease, therefore, must involve more than the viral envelope protein genes and, in the case of dengue, may be associated with specific host factors.

Virulence of poliovirus can be altered by single nucleotide change in the 5'-noncoding region (Kawamura *et al.*, 1989; Racaniello and Meriam, 1986). Multiple amino acid changes in the poliovirus capsid proteins also affect neurotropism of the virus (Westrop *et al.*, 1989; Nomoto *et al.*, 1987; Moss *et al.*, 1989). Sindbis virus virulence can be decreased by single nucleotide changes in the E2 envelope protein (Davis *et al.*, 1986). Recent studies with Sindbis virus infectious clones suggest that multiple genetic changes in both E1 and E2 envelope genes are involved in alphavirus virulence (Lusting *et al.*, 1988). Passage of Ross River virus in mice resulted in increased virulence and sequence changes in one or two nonconservative amino acids in the E2 protein (Meek *et al.*, 1989). These changes occurred in a neutralization determinant which altered the kinetics of virus entry into cells. It is clear that the phenotypic characteristics of RNA virus neurotropism

and virulence are multifactorial, although single amino acid changes in critical genes do affect virulence.

Many of the genome changes which resulted in amino acid substitutions in the SA-14-14-2 proteins are not present in the three virulent JE virus strains compared. Some of these changes are thought to be in critical positions within the proteins and could play an important role in virus attenuation. Functional changes resulting from changes in amino acids within individual proteins can only be appreciated when their role in virus replication is understood. These experiments must await the development of an infectious clone to prepare recombinant viruses for evaluation of genetic changes which affect virus neurovirulence.

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Preclinical neurovirulence studies

A. Comparison of the susceptability of Rhesus monkey and mouse to Japanese encephalitis virus.
[Internal report of the National Institute for the Control of Pharmaceutical and Biological Products, CHINA]

B. Neurovirulence test of Japanese encephalitis vaccine. Histopathology report by James B. Moe, DVM, PhD.

C. Tabular summary of comparitive neurovirulence of attenuated SA14-14-2 and parent SA14

A

COMPARISON OF THE SUSCEPTIBILITY OF RHESUS MONKEY AND MOUSE TO JAPANESE ENCEPHALITIS VIRUS

Ling Jingping[1], Zhu Yingeng[1], Du Guizhi[2], Laiig Shuhui[1], Jia Lili[1], Yu Yongxin[1], Zhou Tiequn[2]

It is well know that the Rhesus monkey is an ideal model for testing neurovirulence of Japanese Encephalitis virus (JE virus), but monkeys are too expensive to be used in our routine work. In this paper we compared the susceptibility of monkey and mouse to JE virus and have found the latter is more susceptible. The data obtained indicate that using mice for neurovirulence tests is more economical and convenient.

Experiment and Discussion

1. Susceptibility of Rhesus monkey and mouse to JE SA14 virulent strain:

For comparison, monkeys and mice were divided into intracerebral (ic) groups and subcutaneous (sc) groups. Monkeys were inoculated with 10^{-2} and 10^{-4} to 10^{-7} dilutions of SA14 virus (original virus titer 6.15 x 10^8 PFU/ml). For each dilution 2 monkeys were used, except for the 10^{-4} where only one was used. Inoculations were in bilateral thalamuses (each 0. 5ml) and lumbar spinal cord (0.2ml). Monkeys were kept for observation and killed with ether before death for JE. Another 3 monkeys were inoculated with 2ml of a 10^{-2} dilution of SA14 subcutaneously and killed on day 18 post infection (PI).

Mice(12-14 gm) were infected intracerebrally (0.03ml each) with 10^{-4} , 10^{-6} , and 10^{-8} dilutions of SA14. Eight mice were used for each dilution and killed before death from JE with ether. Another 30 mice were infected 0.1ml of 10^{-1} dilution of SA14 subcutaneously. A fraction of the 30 mice were killed periodically.

Eight of nine monkeys infected ic died before or on day 8 PI, and all mice infected ic died before or on day 6 PI.

Neuron necrosis was the most characteristic and prominent histopathologic change, mainly distributed in thalamuses, brainstem and spinal cord, particularly in the anterior horn of the cervical spinal cord and substantia nigra of midbrain in monkeys, and cerebral cortex and hippocampus in mice. Inflammatory reaction was not very clearly evident. One monkey, inoculated with 10^{-4} diluted virus (Pathology No. 38643) was still alive on day 18. Its pathologic lesions in the CNS were the same as with other monkeys but less severe.

In the subcutaneous group, no monkeys became sick or died until day 18; pathologic chances were not found in the monkeys' CNS tissues. However, more than half of the mice were sick or died on day 5, and the remainder died by day 14. Pathologic changes, including neuron necrosis and inflammatory reaction were clearly seen, widely distributed in the CNS and more severe in the cerebral cortex than in other areas.

2. Susceptibility to the 14-2 attenuated virus:

Four monkeys were tested by inoculating 0.5ml of 1:5 diluted 14-2 suspension (original virus titer $8x10^6$ PFU/ml) in bilateral thalamuses and 0.2ml in the lumbar spinal cord, and observed until day 18. No clinical signs were seen. Only a minor inflammatory reaction was found, mainly located in the substantia nigra and the cervical spinal cord.

30 mice were inoculated intracerebrally with 0.03ml of a 1:5 dilution of the 14-2 stock. Only a few mice developed some minor clinical signs. None of them died by day 17. Pathologic lesions were limited to the cerebral cortex, hippocampus, and/or the basal ganglia. Compared with SA14, inflammatory reaction was rather evident and neuron necrosis was less severe.

30 mice were inoculated subcutaneously with 0.lml of 1:5 diluted 14-2, observed and killed on day 17. Except for one mouse (No.38751) which showed a few dead nerve cells in cerebral and hippocampus, all mice appeared healthy and no pathologic lesions were seen in CNS tissues.

3. Conclusions

a. When scoring neuron lesions, considering the influences of needle tracks in the thalamuses and lumbar spinal cord of monkeys and the lesion' s location in both monkeys and mice, it is more reliable to choose the anterior horn of the cervical spinal cord and substantia nigra for monkeys, and the cerebral cortex and hippocampus for mice.

b. For mice, whether infected intracerebrally or subcutaneously with the SA14 virulent strain, all developed clinical signs and neuronal lesions. As a result, it is clear that mice are much more susceptible to JE virus than monkeys, and mice therefore can be used in place of monkeys for JE virus neurovirulence tests.

1. National Institute for the Control of Pharmaceutical and Biological Products
2. Beijing Institute of Biological Products

Table 1. Neurovirulence histopathology results of rhesus monkeys inoculated SA14 intracerebrally

Area of CNS	Pathology No. 38637	38638	38639	38640	38641	38642	38643	38651	38652
Frontal Lobe	0[a]	8	0	0	0	0	1	0	0
Parietal Lobe	0	1	0	1	1	0	0	0	0
Temporal Lobe	0	1	0	2	0	0	0	0	0
Occipital Lobe	0	0	0	0	0	0	0	0	0
Hippocampus	0	1	0	?	0	0	0	0	0
Thalamus	2,NT[b]	3	3	3	3	0	3	4,NT	3
Midbrain	2	4	2	2	3	?	2	3	2
Pons	2	2	3	3	3	3	0	2	?
Cerebellum	0	0	0	1	2	2	1	?	1
Medulla oblongate	2	2	?	?	2	1	-[c]	3	3
Cervical Sp. cord	4	4	4	4	3	4	?	4	3
Thoracic Sp. cord	2	2	2	2	3	2	-	2	2
Lumbar Sp. cord	4,NT	3,NT	4,NT	4,NT	4	4,NT	1,NT	4	?
Dilution of virus	10^{-7}		10^{-6}		10^{-5}		10^{-4}	10^{-1}	

a) Neuron lesions: 0= No lesion, 1= 5% of neurons died, 2= 6-20% of neurons died, 3= 21-50% of neurons died, 4= 50% of neurons died.

b) NT= Needle track present

c) -= Tissue not present

Table 2. Neurovilurence histopathology results of mice infected SA14 intracerebraly

Area of CNS	Pathology No. 38656	38657	38658	38659	38661	38662	38663	38664	38666	38667	35668	38669	38670	38676	38677	38671	38672	38673
Cerebral cortex	1	2	3	3	3	2	1	2	4	2	3	3	4	4	4	3	4	4
Hippocampus	4	3	4	4	2	2	1	2	4	2	3	3	4	4	4	3	4	4
Basal ganglia	--	-	1	2	2	1	1	2	-	1	2	-	3	4	2	-	4	4
Thalamus	1	2	2	2	1	-	1	2	1	2	1	2	3	4	2	2	3	-
Midbrain	1	2	2	1	1	1	1	2	1	1	1	2	-	3	2	3	4	4
Pons	0	0	1	1	1	1	1	3	0	0	0	1	1	3	2	2	2	2
Cerebellum	0	0	0	0	0	0	0	0	0	0	0	-	0	1	-	0	0	0
Medulla oblongate	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	2	1	1
Cervical Sp. cord	0	1	0	0	0	0	2	2	0	0	0	2	0	2	1	2	1	0
Thoracic Sp. cord	0	2	0	0	-	0	1	-	0	0	-	-	-	1	-	-	0	0
Lumbar Sp. cord	0	1	0	0	1	1	2	2	0	0	0	1	2	2	1	0	1	0
Dilution of virus	10^{-4}				10^{-6}				10^{-7}							10^{-8}		

Table 3. Neurovilurence histopathology results of mice infected SA14 subscutaneously

Area of CNS	Pathology No. 38681	38682	38683	38684	38685	38686	38687	38688	38689	38690	38691	38692	38693	38694	38695	38696
Cerebral cortex	0	0	0	4	4	4	4	0	3	0	4	4	2	3	3	4
Hippocampus	0	0	0	3	2	-	3	0	0	0	0	1	0	2	0	2
Basal ganglia	0	0	0	3	4	3	3	0	2	0	3	3	1	3	-	-
Thalamus	0	0	-	4	4	2	4	0	2	0	2	-	0	1	2	0
Midbrain	0	0	0	-	3	3	4	0	-	-	2	1	0	1	1	1
Pons	0	0	0	2	2	2	1	0	1	0	1	1	0	1	0	0
Cerebellum	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
Medulla oblongate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cervical Sp. cord	0	0	2	0	0	0	1	0	0	3	0	2	0	2	0	0
Thoracic Sp. cord	-	0	3	0	1	-	-	-	0	2	0	-	0	2	0	0
Lumbar Sp. cord	0	0	2	0	0	0	1	0	0	3	0	2	0	0	0	0
Day	3					5		6		7		8		10	12	14

Table 4. Neurovirulence histopathology results of mice infected 14-2 attenuated vaccine intracerebraly

Area of CNS	Pathology No. 38711	38712	38713	38714	38715	38716	38717	38718	38719	38720	38721	38722	38723	38724	38725	38726	38727	38728	38729	38730	38731	38732
Cerebral cortex	0	0	0	0	0	0	0	1	1	0	2	0	0	1	0	0	0	0	0	0	0	0
Hippocampus	0	0	0	0	0	1	0	2	2	2	2	0	2	1	0	2	0	2	0	0	3	3
Basal Ganglia	0	0	0	0	0	0	0	0	0	0	0	0	0	2	-	0	-	0	0	2	2	0
Thalamus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	0	0	0	0	0	0	0
Midbrain	0	0	0	0	0	-	0	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pons	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cerebellum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	0	0	0	0
Medulla oblongate	0	0	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	0	0	0	-	0
Cervical Sp. cord	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	0	0	0
Thoracic Sp. cord	0	0	0	0	0	0	0	0	0	0	0	0	0	-	6	-	0	0	0	0	0	0
Lumbar Sp. cord	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Day	3		5			6			8			10			12			14			17	

Table 5. Neurovilurence histopathology results of mice infected 14-2 vaccine subscutaneously

Area of CNS	Patholgy No.																							
	38746	38747	38748	38749	38750	38751	38752	38753	38754	38755	38756	38757	38758	38759	38760	38761	38762	38763	38764	38765	38766	38767
Cerebral cortex	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hippocampus	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Basal ganglia	0	0	0	0	0	0	-	-	0	0	-	0	0	0	-	0	0	0	-	0	0	-
Thalamus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Midbtain	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	0	0	0	0	-
Pons	0	0	0	-	0	0	0	0	0	0	0	0	0	0	0	0	-	0	0	0	0	0
Cerebellum	0	0	0	-	0	0	0	0	0	0	0	0	0	0	0	0	-	0	0	0	0	0
Medulla oblongate	-	-	-	-	-	0	-	0	-	0	0	0	-	0	-	-	-	0	-	0	-	-
Cervical Sp. cord	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Thoracic Sp. cord	0	0	0	0	0	0	0	0	0	-	0	0	0	-	0	0	0	0	0	0	0	0
Lumbar Sp. cord	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Day	3		5		7			8			10			12			14			17		

Neurovirulence Test of Japanese Encephalitis Vaccine

Pathology Narrative Comments:

Histopathologic preparations of central nervous system (CNS) tissues from seven Rhesus monkeys were examined. These monkeys had been inoculated in the thalamus and lumbar spinal cord with one of the following strains of Japanese Encephalitis (JE) virus: PDK 14-2 attenuated vaccine (N = 2); HKC 14-2 attenuated vaccine (N = 1); 5-3 attenuated vaccine (N = 2); or SA-14 HKC virulent virus. The following CNS sites were examined: frontal lobe; parietal lobe; temporal lobe; occipital lobe; thalamus (inoculation site); midbrain; pons; cerebellum; medulla oblongata; cervical spinal cord; thoracic spinal cord; and lumbar spinal cord (inoculation site). A scoring system devised for neurovirulence testing of Dengue Viruses was used to evaluate the lesions in the CNS tissues from these monkeys. The results of histopathologic examination are presented in the Individual Animal Neurovirulence Histopathology Results and Summary Tables.

Needle tracks were present in the thalamus of all monkeys and needle tracks or inflammatory lesions were evident in the lumbar spinal cord of all monkeys indicating that each had received a valid inoculation. Although the numbers of animals in each group were limited to one or two per group, some general conclusions regarding neurovirulence were evident. The neurovirulence lesions in the monkeys inoculated with either the PDK 14-2 or the HKC 14-2 attenuated vaccines were all of a minimal grade of severity (V-1) and mainly limited to the anatomic vicinities of the injection sites (thalamus and lumbar spinal cord). The CNS lesions in the monkeys inoculated with the 5-3 attenuated vaccine were quite similar in distribution and severity to those described in the monkeys receiving the 14-2 strains, except for a V-2 (more severe) lesion in the temporal cortex of one monkey. Therefore, based on these limited observations, the three attenuated vaccines (PDK- 14-2, HKC 14-2, 5-3) appeared to have similar, low potential for neurovirulence.

.The neurovirulence lesions were clearly more severe and widely distributed in the two monkeys inoculated with the virulent SA-14 HKC virus than in those inoculated with attenuated vaccines. In the local areas around the injection sites the neurovirulence lesions were more extensive and there were frequently lesions located in other areas (mid brain, pons, temporal lobe, parietal lobe, frontal lobe, cervical spinal cord, thoracic spinal cord) remote from the inoculation sites. The pattern of lesion distribution indicated that the SA-14 HKC virulent virus infection had disseminated more widely throughout the CNS than was the case with the attenuated viruses used for vaccines.

The lesions in monkeys inoculated with virulent virus and with any of the attenuated virus vaccine strains were characterized mainly by the perivascular lymphocytic cuffs and focal mononuclear cell infiltration ("glial nodules"). Rarely was there direct evidence of direct neuron damage (degeneration or necrosis).

Based on the results of this limited study, it appears that the neurovirulence potential of the PDK 14-2, HKC 14-2, and 5-3 attenuated vaccines is quite low and acceptable for continued development. Ideally this study would be extended to involve more (N = 10) monkeys inoculated with the vaccine strain intended for widespread clinical use and a control group (N = 2) inoculated with uninfected culture fluids. This extension of the study would provide more definitive data regarding safety and neurovirulence potential of the proposed vaccine.

James B, Moe, DVM, PhD

COMPARATIVE NEUROVIRULENCE OF ATTENUATED SA14-14-2 AND PARENT SA14

			3-week-old Mice		Adult Rhesus monkeys	
Virus strain (Virus titer, PFU/ml)	Inoculation route	Dilution	Died /tested	Histopathologic score (neuronal lesions)[1]	Died /tested	Histopathologic score (neuronal lesions)[1,2]
SA14 parent ($6.15x10^8$)	IC	10^{-1}	ND	ND	2/2	2-4
		10^{-4}	8/8	2-4	0/1	2-3
		10^{-5}	ND	ND	2/2	2-4
		10^{-6}	8/8	2-3	2/2	2-4
		10^{-7}	8/8	2-4	2/2	2-4
		10^{-8}	8/8	2-4	ND	ND
	SC	10^{-1}	30/30	2-4(day 5)	ND	ND
SA14-14-2 ($8x10^6$)	IC	1:5	0/30[3]	0-2	0/4	0-1
	SC	1:5	0/30[3]	0(1)[4]	ND	ND

INDIVIDUAL ANIMAL NEUROVIRULENCE HISTOPATHOLOGY RESULTS TABLE

Pathology No. (Inoculum)

Area of CNS	37756 (PDK14-2)	37757 (PDK14-2)	37758 (HKC14-2)	37759 (5-3)	37760 (5-3)	37761 (SA14HKC) Virulent	37762 (SA14HKC Virulent
Frontal Lobe	0[b]	0	V-1[d]	0	--[e]	0	V-1
Parietal Lobe	NT[c]	0	0	0	NT	NT	V-1,NT
Temporal Lobe[a]	0	V-1	0	V-2	V-1	V-1,NR	V-2
Occipital Lobe	0	0	0	0	0	0	0
Thalamus	V-1,NT,NR	V-1,NT	V-1,NT	V-1,NT	V-1,NT	V-1,NT,NR	V-2,NT
Midbrain	0	V-1	V-1	0	0	V-2	V-2
Pons	0	0	0	0	--	--	V-2
Cerebellum	0	0	0	0	0	NR	V-1,NR
Medulla Oblongate	--	0	--	--	--	--	--
Cervical Sp. Cord	0	0	0	0	--	--	V-1
Thoracic Sp. Cord	0	0	0	0	0	V-2	V-2
Lumbar Sp. Cord	V-1,NT,NR	NT	V-1,NT	V-1,NR	V-1,NT,NR	V-2,NT	V-2,NT

[a] Temporal section includes hippocampus and basal ganglia
[b] 0 = No lesions
[c] NT = Needle track present
[d] V-1, V-2, V-3, V-4 = Neurovirulence lesions, V-1 is least severe, V-4 is most
[e] -- = Tissue not present

SUMMARY TABLE OF NEUROVIRULENCE HISTOPATHOLOGY LESIONS-INCIDENCE AND SEVERITY

Area of CNS	PDK		14-2		HKC		14-2[c]		5-3		SA-14 HKC Virulen	
	Prop.[a] Affected	Severity Score[b]	Prop. Affected	Severity Score	Prop. Affected	Severity Score	Prop. Affected	Severit Sco				
Frontal Lobe	0	--	0.5	0.5	--	--	0.5	0.5				
Parietal Lobe	0	--	0	--	0	--	0.5	0.5				
Temporal Lobe	0.5	0.5	0	--	1.0	1.5	1.0	1.5				
Occipital Lobe	0	--	0	--	0	--	0	--				
Thalamus	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.5				
Midbrain	0.5	0.5	1.0	1.0	0	--	1.0	2.0				
Pons	0	--	0	--	0	--	1.0[b]	2.0				
Cerebellum	0	--	0	--	0	--	0.5	0.5				
Cervical Spinal Cord	0	--	0	--	0[b]	--	1.0[b]	1.0				
Thoracic Spinal Cord	0	--	0	--	0	--	1.0	2.0				
Lumbar Spinal Cord	0.5	0.5	1.0	1.0	1.0	1.0	1.0	2.0				

[a] Proportion Affected (0.5 = 50%, 1.0 = 100%)
[b] Severity Score = average of all animals, affected and non-affected
[c] Includes data for only 1 animal

Major published clinical studies and summary of additional clinical information

- Immunogenicity of live attenuated SA14-14-2 Japanese encephalitis vaccine- a comparison of 1- and 3- month immunization schedules
  J Inf Dis 177: 221-23, 1998

- Short term safety of live attenuated Japanese encephalitis vaccine (SA14-14-2): results of a randomized trial with 26,239 subjects
  J Inf Dis 176: 1366-1369, 1997

- Effectiveness of live-attenuated Japanese encephalitis vaccine (SA14-14-2): a case-control study
  Lancet 347: 1583-1586, 1996

- Summary table of efficacy studies in China from 1988 through 1992

- Primary and booster immune response to SA14-14-2 Japanese encephalitis vaccine in Korean infants
  Vaccine: to be published

Effectiveness of live-attenuated Japanese encephalitis vaccine (SA14-14-2): a case-control study

Sean Hennessy, Liu Zhengle, Theodore F Tsai, Brian L Strom, Wan Chao-Min, Liu Hui-Lian, Wu Tai-Xiang, Yu Hong-Ji, Liu Qi-Mau, Nick Karabatsos, Warren B Bilker, Scott B Halstead

Summary

Background Japanese encephalitis is a major cause of death and disability throughout Asia, including the Indian subcontinent. Although an effective vaccine for Japanese encephalitis is available, hundreds of millions of susceptible individuals remain unimmunised because of the vaccine's cost. In 1988, an inexpensive live-attenuated vaccine (SA14-14-2) was licensed in China. We have measured the effectiveness of this vaccine.

Methods In a case-control study in rural Sichuan Province, China, the 56 cases consisted of children admitted to hospital with acute Japanese encephalitis, and were confirmed serologically. 1299 village-matched and age-matched controls were identified, and vaccination histories obtained from pre-existing written records.

Findings The effectiveness of one dose was 80% (95% CI 44 to 93%); that of two doses was 97·5% (86 to 99·6%). Controlling for multiple potential confounders did not alter these results.

Interpretation We conclude that a regimen of two doses of live-attenuated Japanese encephalitis vaccine, administered 1 year apart, is effective in the prevention of clinically important disease. Subsequent study is needed to assure the safety of this vaccine.

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See Commentary page 1570

Department of Biostatistics and Epidemiology, and Center for Clinical Epidemiology and Biostatistics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA (S Hennessy PharmD, B L Strom MD, W B Bilker PhD); **Clinical Epidemiology Unit, West China University of Medical Sciences, Chengdu, Sichuan, China** (L Zhengle MD, W Chao-Min MD, L Hui-Lian MD, W Tai-Xiang, Y Hong-Ji, L Qi-Mau MSc); **Centers for Disease Control and Prevention, Fort Collins, Colorado** (T F Tsai MD, N Karabatsos PhD); **Division of General Internal Medicine, Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia** (B L Strom); **and Rockefeller Foundation, New York, New York, USA** (S B Halstead MD)

Correspondence to: Dr Sean Hennessy, Center for Clinical Epidemiology and Biostatistics, University of Pennsylvania School of Medicine, Room 832 Blockley Hall, Philadelphia, PA 19104, USA

Introduction

Japanese encephalitis is an acute central-nervous-system infection that occurs over a vast geographic area (including India, China, Japan, and virtually all of Southeast Asia). The disease causes 35 000 cases of encephalitis and 10 000 deaths each year, and about 30% of survivors develop serious permanent sequelae.[1] Japanese encephalitis is an RNA flavivirus transmitted by *Culex tritaeniorhynchus* mosquitoes. Swine, birds, and other vertebrates are amplifying hosts. In most regions, the disease is seasonal, with most cases appearing between May and September. Japanese encephalitis is more common in rural than in urban areas, because the vector mosquito breeds in rice paddies and other standing water.[1] Because immunity to the disease is acquired naturally over time, Japanese encephalitis is primarily a childhood disease, with most cases occurring before age 15. The incidence is also elevated in the elderly, possibly because of waning immunity.

The incidence of Japanese encephalitis has declined greatly over the past three decades, presumably because of the widespread use of vaccines and because of mosquito-control efforts.[2] Inactivated weanling-mouse brain-derived vaccine is manufactured by Biken (Japan) and distributed internationally by Pasteur Merieux and Connaught Laboratories. In addition, a limited amount of a killed Japanese encephalitis vaccine is available from Green Cross (Korea). The efficacy of a two-dose regimen of the Biken vaccine was 91% (95% CI 70 to 97%) in a randomised controlled trial.[3] Currently, a three-dose regimen is recommended in the US,[4] where the average wholesale price for three doses is $147.[5] Because of the cost of the currently available vaccine, many countries in endemic areas cannot implement large-scale immunisation.

In 1988, the Chinese National Institute for the Control of Pharmaceutical and Biologic Products approved a live-attenuated, primary baby-hamster-kidney-cell-derived vaccine for Japanese encephalitis that is produced from the SA14-14-2 viral strain.[6] This vaccine is manufactured by the Chengdu Biological Products Institute, and in some provinces has replaced the older, killed cell-culture vaccine because of the newer vaccine's higher putative efficacy (98%[1] versus 78%[7]) and slightly lower production costs (US$0·02–0·03 versus 0·03–0·04 per dose[1]). The Chengdu Biological Products Institute says that the vaccine has been administered to over 100 million Chinese children. Although the safety of this vaccine with respect to common adverse events has been shown in a study of 1026 vaccinated children,[6] its safety with respect to rare events has not been studied. Non-randomised field trials in China suggest the efficacy of a single dose of the live-attenuated vaccine may be approximately 95%, and that of two doses one year apart may exceed 98%.[1]

However, the role of confounding in these non-randomised studies is unknown. We aimed to replicate these findings while controlling for potential confounders.

Patients and methods

Study design

We conducted a case-control study of incident cases of Japanese encephalitis in rural areas surrounding the city of Chengdu, Sichuan Province, China. Our overall strategy was to identify incident cases and to compare their vaccination histories with those of non-diseased controls. The study was approved by the University of Pennsylvania's Committee on Studies Involving Human Beings.

Sichuan Province

Sichuan, in southwestern China, is the largest and most populous Chinese province, with a 1990 population of 106 370 000. The Dujiangyan irrigation on the Min River irrigates 3 million hectares of rice and wheat crops, a vast expanse of flooded fields, which are the favoured habitat of *C tritaeniorhynchus*. As a result, the annual incidence of Japanese encephalitis is estimated by Sichuan Province public-health officials to be 1 per 100 000, despite an existing vaccination programme.

Nearly all inhabitants of rural Sichuan are subsistence farmers, organised into geographically and economically homogeneous agricultural villages. Each village is associated with a government-supported county hospital that meets the inpatient needs of villagers. In addition, each village is served by a government-supported county anti-epidemic station that provides centralised support for village-run immunisation campaigns.

Children in rural Sichuan are immunised with Japanese encephalitis vaccine at 1 and 2 years of age, and receive a booster at 6. Immunisation occurs during annual campaigns each spring, with vaccine that was produced earlier that year. No other vaccine is given concurrently. Vaccines are administered by the village doctor, who provides primary health-care for that village and maintains a village registry that includes documentation of all immunisations. The structure of these registries is specified by the Province, with children being entered shortly after birth. Although parents are charged a small fee of 2 yuan (US$0·24, typically 0·02–0·05% of an annual rural household income) to have a child immunised against Japanese encephalitis, it is believed that absence from the village during the brief vaccination campaign, rather than inability to pay this small sum, is the major reason that vaccination campaigns miss some children.

Identification of cases

One of us (LZ) recruited and trained physicians and staff from twenty-four country hospitals and two urban hospitals that serve rural areas surrounding urban Chengdu (the location of the co-ordinating centre, West China University of Medical Sciences [WCUMS]) to identify suspected Japanese encephalitis cases. Suspected cases were identified during the period of peak transmission (May 1 to Sept 30) of 1993, and consisted of all children under 15 who presented to one of the participating hospitals with acute central-nervous-system findings (headache, lethargy, ataxia, delirium, seizure, or coma) accompanied by a temperature of 37·5° or higher. Potential cases were enrolled without regard to immunisation status. For serological confirmation, we enrolled only suspected cases who had at least one serum or cerebrospinal-fluid (CSF) sample drawn as part of clinical care. Clinicians were asked to obtain a convalescent serum sample when possible.

Serological confirmation

Serum and CSF specimens were kept cold and transported to WCUMS, where they were stored at −20°C until samples were shipped to the Centers for Disease Control and Prevention laboratory in Fort Collins, Colorado, USA. Neutralising antibodies against Japanese encephalitis were measured with a plaque-reduction test.[8] Cases satisfying the clinical criteria were classified as presumptive Japanese encephalitis if the serum neutralising-antibody-titre was 80 or higher and as confirmed Japanese encephalitis if paired serum samples demonstrated a fourfold change in neutralising antibody titre or the CSF neutralising antibody titre was 10 or more.[8]

Controls

To select controls, investigators travelled unannounced to the village of residence of each confirmed case, where they located the village doctor, and examined the village registry. Controls were identified from this registry, and consisted of all children listed on the village registry who were born the same year as the case, and who did not develop clinical encephalitis during the study period. Appearance on the village registry was also an inclusion criterion for cases. Because each village experienced only one case during the study period, the control group consisted of all children of a given age who were at risk of developing disease at the time that the case occurred. This scheme is termed density sampling.[10]

We matched on village (rather than region-wide population-based sampling) for two reasons. First, because there is no overall roster of children living in the region, identifying unmatched population-based controls was not feasible. Second, we wished to avoid potential confounding that would occur if vaccination programmes tended to focus on villages with a high concentration of infected vectors, which could create an artificial positive association between vaccination and disease. We enrolled all potential controls, rather than sampling among them, because sampling would have required more effort than including all controls yet would have resulted in somewhat less statistical power.

Vaccination history

Vaccination histories were obtained from village registries for cases and controls. Because pilot experience revealed that the quality of vaccination records was variable among villages, a subjective score, ranging from one (poor) to five (good), was assigned to the records of each village, based on the impression of researchers from WCUMS.

Statistical analysis

In randomised trials, vaccine efficacy is calculated as 1-RR, where RR is the rate ratio, or the incidence of disease in the vaccinated group divided by the incidence in the unvaccinated group. When density sampling is used in a case-control study, the exposure odds ratio (OR) is an unbiased estimate of RR.[10] Therefore, vaccine effectiveness was calculated as 1-OR.[11]

We used conditional logistic regression[12] to calculate ORs and 95% CIs, conditioned on matched set, for one, two, and three doses of live-attenuated vaccine, each compared with zero doses. We used multivariate conditional logistic regression to evaluate and control for the effects of potential confounding variables. In the primary analysis, we evaluated the effectiveness of two versus zero doses. We used EGRET.

Results

158 cases of potential Japanese encephalitis were identified clinically and after having had at least one biological sample drawn. 59 potential cases (37%) were excluded because the serological evidence for recent infection with Japanese encephalitis virus did not support classification as presumptive or confirmed. 39 (25%) potential cases were serologically presumptive and therefore excluded from the analysis of vaccine effectiveness. 60 potential cases (38%) were serologically confirmed.

Doses received	Cases (n=56)	Controls (n=1299)	Effectiveness (95% CI)
0	38 (68%)	615 (47%)	..
1	11 (20%)	332 (26%)	80% (44 to 93%)
2	6 (11%)	308 (24%)	97·5% (86 to 99·6%)
3	1 (2%)	44 (3%)	NE

NE=not evaluable because of insufficient data.

Table: **Effectiveness of live-attenuated Japanese encephalitis vaccine (SA14-14-2)**

52% of confirmed cases were female, and the mean age of all cases was 4·7 years. Eligible controls were unavailable for four confirmed cases. For the remaining 56 confirmed cases, 1299 matched controls were identified. Controls' average age was also 4·7 years; 57% of controls were female.

Previous vaccine exposure among cases and controls is shown in the table. 68% of cases received no vaccine, compared with 47% of controls. The effectiveness of one dose of the vaccine was 80% (95% CI 44 to 93%), and that of two doses was 97·5% (86 to 99·6%). Simultaneously adjusting for gender and past receipt of killed vaccine, effectiveness of one dose of live-attenuated vaccine was 71% (21 to 90%), and that of two doses was 97·6% (86 to 99·6%). Effectiveness was similar in boys and girls (p for interaction=0·35).

After exclusion of subjects who had received any killed vaccine, effectiveness for one dose was 61% (−14 to 86%), and for two doses was 97·5% (86 to 99·6%). For only subjects whose village had a vaccination-record quality-score of five (ie, those with the best vaccination records), effectiveness of one dose was 82% (12 to 96%), and that of two doses was 94% (16 to 99%). Because few subjects received three doses, the effectiveness of three doses could not be estimated.

Discussion

Given the evidence for the immunogenicity and efficacy of live-attenuated Japanese encephalitis vaccine,[1] and the known safety and efficacy of the Biken vaccine,[3] we decided that a placebo-controlled trial of SA14-14-2 vaccine would have been unethical. Therefore we did a case-control study to provide the best estimate of vaccine effectiveness.

Our data provide strong evidence for the vaccine's effectiveness. The effectiveness of a single dose was 80% (95% CI 44 to 93%), while that of two doses was 97·5% (86 to 99·6%). The observed 80% effectiveness of a single dose is lower than the 95% efficacy rate in prospective non-randomised trials.[1] This difference may have arisen because previous studies were conducted in more highly endemic areas than our study area; therefore, the immunity provided by a single vaccine dose in such a setting may have been reinforced by either previous or subsequent natural exposure to Japanese encephalitis virus. This explanation is supported by previous immunogenicity studies of inactivated Japanese encephalitis vaccine in which two doses produced adequate immunity among persons from areas with transmission of this disease, but three doses were necessary for persons from non-endemic areas.[1,13] Alternative explanations for the apparent difference in the effectiveness of a single dose include inadequate refrigeration or improper administration technique during routine immunisation campaigns, which would have been reflected in our results, and unmeasured differences between vaccinated and unvaccinated groups in earlier studies. The apparent superiority of two doses compared with one dose is consistent with previous immunogenicity studies.[1,6]

The 97·5% effectiveness estimate is not directly evident from the proportion of vaccine exposure in the cases (table), because 13% of cases received two or more doses. However, that frequency (13%) is substantially lower than the proportion of people in the general population who have received two or more doses which, based on the control series, can be estimated to be 27%.

To interpret our study, we must look for possible bias or confounding. Selection bias would have occurred if cases were chosen on the basis of vaccination status or if they did not truly have the disease of interest. We believe that the use of an organised surveillance system and an explicit clinical case definition, combined with a rigorous serological case definition, served to minimise this possibility. Because controls should represent the population of individuals who would have been included as cases had they developed disease,[14] and because there is universal access to government-supported hospitals in rural Sichuan, the use of matched population-based controls should minimise the possibility of bias in the selection of controls. Information bias occurs in a case-control study when exposure status is misclassified differentially between cases and controls. We attempted to avoid this bias by relying on the same pre-existing written records to ascertain the exposure status of cases and controls.

A confounder is a factor that is associated with the exposure of interest and the outcome of interest to artificially inflate or deflate the true association. In Japanese encephalitis, the known determinants of disease are age, sex, vaccination history, and exposure to infected vectors. We accounted for potential effects of age by matching on year of birth and performing the corresponding matched analysis. We controlled for sex and past receipt of killed vaccine by stratified and multivariate analyses. We matched on village (and did the corresponding matched analysis) to control for exposure to infected mosquitoes, and for potential confounders (eg, socioeconomic status) that might operate through this mechanism. To do so, we used residence in a particular village (within age and sex strata) as a proxy for factors determining exposure to infected vectors. To the degree that this proxy does not capture differences in exposure to infected vectors and that these differences are associated with vaccination status, residual confounding may remain. However, we believe that the magnitude of any such residual confounding is probably small.

Provided that a case-control study is free from bias and confounding, it may offer some advantages over a randomised controlled trial. First, it provides a measure of the clinical effectiveness of the vaccine as actually used, whereas most randomised controlled trials measure the efficacy of the vaccine under carefully controlled experimental conditions, and do not reflect the effects of breaches of acceptable protocol, such as inadequate refrigeration, that may occur in usual practice. A second advantage is that an unbiased case-control study can provide a more precise estimate of vaccine effectiveness (ie, narrower 95% CIs) than could have been achieved by a similarly sized randomised trial.

Our study was not designed to measure the safety of live-attenuated Japanese encephalitis vaccine. However, since the principal safety concern about this live vaccine is

the possibility of inadequate attenuation and the resultant possibility of vaccine-induced encephalitis, our observation of a negative association between vaccination and hospital admission for Japanese encephalitis supports previous observations of the vaccine's safety.[9] Nevertheless, further study is needed to assure the safety of this vaccine with respect to other adverse events.

In conclusion, we conducted a case-control study of incident cases of hospital admission for Japanese encephalitis to measure the effectiveness of live-attenuated hamster-kidney-cell-derived Japanese encephalitis virus (SA14-14-2). A regimen of two doses of vaccine administered 1 year apart prevented clinically important disease. If the safety of this vaccine is confirmed in a sufficiently large study, and it is made available internationally at an affordable price, the widespread administration of SA14-14-2 vaccine could prevent thousands of deaths and cases of permanent disability each year throughout Asia.

We thank the many clinicians and public-health officials that contributed to this study, and Wang Jialiang, Director of the Clinical Epidemiology Unit of West China University of Medical Sciences, for his administrative support. We also thank the Rockefeller Foundation (RF92085, #26 and RF 92085, #27) for financial support.

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Functional activation of mutant human insulin receptor by monoclonal antibody

Anna Krook, Maria A Soos, Sudhesh Kumar, Kenneth Siddle, Stephen O'Rahilly

Summary

Background A mutant insulin receptor, Ser323Leu, has been reported in two severely insulin-resistant patients with Rabson-Mendenhall syndrome. In both cases, extreme hyperglycaemia could not be controlled by conventional antidiabetic therapy. The Ser323Leu mutant insulin receptor is inserted normally in the plasma membrane but has very low binding affinity for insulin. A monoclonal antibody directed against the extracellular domain of the insulin receptor (83.14) can mimic the natural ligand as far as the first step after ligand binding—autophosphorylation of the intracellular domain of the receptor. We have investigated whether antibody binding can imitate autophosphorylation of the Ser323Leu mutant receptor and lead to metabolic events within the cell.

Methods The effects of insulin and the insulin-receptor monoclonal antibody on receptor autophosphorylation and glycogen synthesis were compared in Chinese hamster ovary cells expressing the wild-type human insulin receptor, mock-transfected cells, cells expressing an insulin-receptor mutant without autophosphorylation capacity, and cells expressing the Ser323Leu mutant receptor.

Findings Cells expressing the Ser323Leu mutant receptor had very low specific insulin binding and, unlike cells expressing wild-type insulin receptors, did not show autophosphorylation or stimulation of glycogen synthesis in response to insulin. However, exposure of cells expressing the Ser323Leu mutant receptor to monoclonal antibody 83.14 resulted in autophosphorylation and stimulation of glycogen synthesis similar to that seen in cells expressing wild-type insulin receptors.

Interpretation Although insulin does not bind to cells expressing the Ser323Leu mutation, insulin signalling can be mimicked by exposure of the cells to an antibody to the extracellular domain of the insulin receptor. Activation by monoclonal antibodies of mutant transmembrane receptors that show normal cell-surface expression but defective ligand binding may provide an approach to the therapy of some subtypes of inherited hormone resistance for which little effective treatment is available.

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Departments of Medicine (A Krook PhD, M A Soos PhD, Prof S O'Rahilly MD) **and Clinical Biochemistry, University of Cambridge** (A Krook, M A Soos, Prof K Siddle PhD, Prof S O'Rahilly), **Addenbrooke's Hospital, Cambridge CB2 2QR, UK; and Department of Medicine, Queen Elizabeth Hospital, Birmingham** (S Kumar MD)

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Introduction

To act at a cellular level, circulating hormones and cytokines require receptors that cross the cell membrane.

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Japanese encephalitis: a Chinese solution?

See page 1583

Health professionals who work outside of Asia may not be familiar with the clinical characteristics and epidemiology of Japanese encephalitis (JE) and therefore may not immediately grasp the implications of the paper by Hennessy et al, in this week's *Lancet*. JE is a vaccine-preventable disease caused by an RNA flavivirus found only in Asia in a broad band extending from India to Japan and from China to Papua New Guinea (figure). This region has a population of about 2·8 billion, with 72 million births per year.[1] Seroprevalence studies in endemic regions indicate that antibodies are acquired from infancy and reach nearly universal proportions by adulthood. Although most JE infections are symptomless, encephalitis occurs in 1–20 of every 1000 infections.[2] There is no effective or specific treatment for the encephalitis, which has a mortality of 20–40%, and produces residual neurological and psychiatric sequelae in 25–40% of survivors. It is likely that the disease is grossly underreported: approximately 50 000 cases are reported annually from the affected areas of Asia, with incidences of 1 to 10 per 10 000.[2] Overall, JE has major economic consequences in Asia, although these do not seem to have been fully evaluated.[3]

Most authorities agree that control of JE requires universal childhood immunisation, because 70% of reported cases occur in young children, and control of the mosquito vectors will be difficult to achieve in endemic regions, many of which are characterised by rice paddy cultivation.[4] In response to the impact of this disease, Japanese and Chinese scientists have developed effective vaccines.

The vaccine developed by Japanese scientists is a formalin-inactivated Nakayama-Yoken JE virus, which is produced in mouse brain. This vaccine has been available commercially since 1968 at a reported wholesale cost in Asia of approximately US$2.30 per dose.[3] It has been widely used in Japan, and a prospective controlled trial in Thai schoolchildren showed an efficacy of 91%.[3] This vaccine was licensed by the US Food and Drug Administration in 1993.[5] Variants of this vaccine are manufactured in Thailand, India, Taiwan, and Korea.[6,7] Because it is produced by cerebral injection of infant mice, it is laborious to manufacture and "there is reason to doubt, based on past performance, that output of vaccine will be sufficient for national needs".[3] Though generally regarded as safe, there have been rare reports of anaphylactic reactions with the use of this vaccine in travellers from Australia, Europe, and North America.[5]

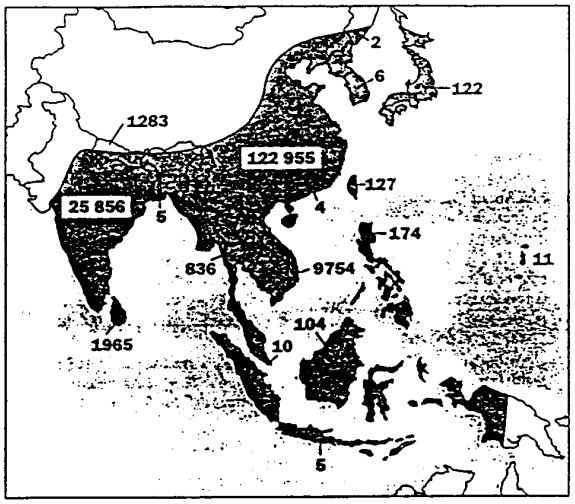

Figure: **Reported cases of JE, 1986–90, and regions of proved or suspected enzootic viral transmission.**

Although development of a live vaccine from a neurotropic encephalitogenic virus is a daunting task, Chinese scientists have developed a live attenuated vaccine from JE virus strain SA 14–14–2, which was passed through weanling mice and is produced in primary baby hamster kidney cells (an unconventional cell-line for vaccine production). This vaccine has been shown to be safe and immunogenic[4,8] and has been administered to over 100 million children in vaccine programmes in China. The report in this issue of the *Lancet*, based on a case-control study in Chengdu province, estimates effectiveness among inpatients with JE at 98%, which is welcome news. As noted by the authors, further controlled studies of the safety of the live attenuated vaccine are necessary to allow confidence regarding its widespread use. Its effectiveness and its reported low cost of $0.03 per dose suggest that it might be useful in the WHO Expanded Programme on Immunisation.

Other approaches to vaccine development have been explored; the JE virus genome has been sequenced and protective M, E, and NS1 viral proteins have been characterised. In addition, vector-expressed JE vaccines are being investigated.[4,9] However, there seems little need to await improved vaccines, given the efficacy and cheapness of the live, attenuated vaccine. Nevertheless, if an improved vaccine can be shown to induce protection after a single injection, it may have an advantage over the live-attenuated vaccine, which requires two injections.

What are the next steps to make this live-attenuated vaccine more available to the Asian populations at risk? Its production in standard cell lines used for vaccine production such as Vero or human diploid cells would increase its acceptability for licensing authorities in many countries. Global and Asian public health agencies and vaccine manufacturers should consider the best approach to production and distribution of this cheap and effective weapon against Japanese encephalitis.

Mark C Steinhoff
School of Hygiene and Public Health, Johns Hopkins University, Baltimore, MD, USA

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Influence of physician perceptions on putting knowledge into practice

The introduction of the latest medical discoveries into real-world practice is a challenge. The discontinuity in the application of research to the practice of clinical medicine was well exemplified at the American College of Cardiology's Annual Scientific Session this year when the results of the widely heralded DIG trial were announced. After 200 years of use, digitalis has at last been shown to have no impact on total mortality in patients with congestive heart failure (CHF) but to significantly reduce hospital admission rates and CHF-related mortality. A plenary speaker asked the audience of 5000 cardiologists "How many of you will change your utilisation of digitalis as a result of this trial?" About 50% indicated that they would change their use of the drug and 50% indicated that they would not. Even when cardiologists were presented with statistically significant, powerful data, "buy in" was not assured.

The application of primary-prevention measures depends to a large extent on the doctor's perception of the risk status of the patient and of the value of the preventive measure. Would the perceptions of the generalists differ from those of specialists? Friedman and colleagues[1] attempted to find out whether family physicians, general internists, and cardiologists differed in their perceptions of baseline cardiovascular risk and of the benefit of drug therapy for middle-aged men with hypercholesterolaemia and for elderly persons with isolated systolic hypertension. They also assessed what these groups of doctors thought of the value of coronary bypass graft surgery in patients with stable angina and left main coronary artery stenosis. They found that cardiologists provided lower, more accurate estimates of baseline cardiovascular risk and of absolute therapeutic benefit than did either of the other two groups.

In searching for explanations for the quasi-quantitative responses that they obtained, Friedman and colleagues wondered whether the differences between the physician groups could have been due to generalists forgetting the "numbers" behind their impression of whether a treatment was beneficial or effective; to the research training or narrower focus of cardiologists; or to "ego bias" on the part of generalists, whose perceptions might have been influenced by the view that primary prevention is a defining characteristic of primary care.

Their analysis overlooks the realm of primary care, where generalist physicians take care of the whole individual, using not only medical knowledge but also the history, physical findings, and information acquired from continuing care of the individual to guide them in the management of their patients.

In my experience patients often refuse specific therapeutic measures simply because they are too expensive or they are just not for them. At such moments, I recall Medalie's[2] landmark study of survival among 5000 Israeli men after myocardial infarction. The two factors in the multifactorial analysis that most closely correlated with survival were not blood pressure, cholesterol, or smoking, but the presence of a loving spouse and having a supervisor at work who respected you. The challenge for the generalist is to combine most fully knowledge of the natural history of the disease, of therapeutics, and of his or her patient in a way that results in maximum compliance and success.

Although Friedman et al have examined a very important topic, their study is not without flaws. The response rate was only about 40% which, although not uncommon for some surveys, does not provide a secure base for data analysis. This point is especially true when participants are drawn from a very heterogeneous population—in this case the American Medical Association masterfile. Individuals choose what information they wish to put into the file. In Friedman et al's study, 15 physicians had no return address, and 5 were retired or deceased. Nor was there information on their qualifications, or on their work settings (eg, academic or not). Other data to corroborate the appropriateness of the samples would have strengthened the study. In view of the heterogeneity of the sample, not surprisingly there was a wide range of responses. The range was so wide that medians and quartiles of response were used as the units of statistical measure rather than means and standard deviations.

The challenge of effectively communicating to busy physicians information that could form the basis of rational clinical decision-making is best met by guidelines, practice-oriented texts, and protocols created jointly by generalists and specialists. The use of CD-ROM and electronic media to build point-of-service quality checks into office routines has the potential of cutting through the staggering overload of information confronting physicians. These systems can provide focused guidelines, reminders, and updates. They can be standardised for all generalists and specialists, and be presented in plain English.

John Noble
Section of General Internal Medicine, Boston University School of Medicine, Boston, MA, USA

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Immunogenicity of Live Attenuated SA14-14-2 Japanese Encephalitis Vaccine—A Comparison of 1- and 3-Month Immunization Schedules

T. F. Tsai, Yu Yong-Xin, Jia Li Li, Ravithat Putvatana, Zhang Ran, Wang Shougui, and Scott B. Halstead

Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Ft. Collins, Colorado; Institute for Control of Pharmaceutical and Biological Products, Temple of Heaven, Beijing, and Chengdu Institute of Biological Products Chengdu, China; Naval Medical Research and Development Command Bethesda, Maryland

Live attenuated SA14-14-2 Japanese encephalitis (JE) vaccine has been safe and effective in >100 million immunized children, but its current administration schedule of two doses given a year apart does not lend itself to inclusion in established Expanded Program of Immunization (EPI) schedules of childhood immunization. Immune responses to immunization at shorter intervals were compared in middle-school-aged children immunized with two doses separated by 1 month (n = 116) or 2.5 months (n = 115). Two vaccine lots were compared. Seroconversion to the vaccine was observed in 100% of vaccinees immunized in the 1-month schedule and in 94% (lot 2) and 100% (lot 1) of vaccinees immunized in the 2.5-month schedule. Geometric mean titers were almost 2-fold higher with the longer schedule. The routine administration of JE SA14-14-2 vaccine to infants in an EPI schedule should be possible using either interval.

Japanese encephalitis (JE), a mosquitoborne flavivirus infection, is a leading cause of childhood viral encephalitis in Asia. Childhood immunization with an inactivated mouse brain–derived vaccine has nearly eliminated the disease in Japan, Korea, and Taiwan, but tens of thousands of cases still occur annually in the region, principally because the complexity of purifying the vaccine from infected mouse brains and its cost, about US$1.50 per dose, has limited more widespread distribution in developing Asian countries [1–3]. A live attenuated JE vaccine (SA14-14-2) was licensed in China in 1988 and since then has been given safely to >100 million children in annual spring campaigns [1, 4]. In field trials comprising >250,000 children, two doses given a year apart yielded efficacies >97%. A recent case-control effectiveness study further confirmed that two doses were 98% effective in preventing the disease, while one dose had an effectiveness of 85% [5].

To facilitate the integration of SA14-14-2 JE vaccine into routine childhood immunization schedules, it would be advantageous for the two doses to be administered within a shorter, more convenient interval than 1 year. The following study compared neutralizing antibody responses in children immunized with two vaccine doses given 1 or 2.5 months apart.

Materials and Methods

Study location and subjects. Previous studies had shown better immune responses in vaccinees from JE-endemic areas, presumably reflecting the unmeasured effects of prior inapparent JE or other flavivirus infections. In effect, booster responses were measured in these individuals. In this study, subjects were recruited from Dunhua City, Jilin Province, in extreme northeastern China. At 43° N latitude (approximately that of Hokkaido island, Japan) and in an urban setting, it is highly unlikely that an enzootic JE virus transmission cycle is maintained in this location. Children 12–15 years of age were recruited from middle schools and randomly assigned to the two immunization schedules.

Vaccination and bleeding schedules. Two vaccine production lots made in different cell culture lots were compared, 941010-1 and 941010-2 (abbreviated as lot 1 and lot 2, respectively). Their respective infectious titers were $\log_{10}$ 6.8 pfu/mL and $\log_{10}$ 6.4 pfu/mL. Vaccine was reconstituted with normal saline, as prescribed by the manufacturer (Chengdu Vaccine Production Institute, Chengdu, China). Subjects were divided further into groups receiving normally reconstituted vaccine or a further 1:3 dilution (to calculated $\log_{10}$ infectious titers of 6.3 and 6.0 pfu/mL respectively). Reconstituted vaccine was used immediately.

Immunizations were administered and venous blood samples were obtained as follows: 116 children received two vaccine doses 1 month apart. With lot 1, 31 children received undiluted vaccine and 25 children diluted vaccine; with lot 2, 30 children each received undiluted and diluted vaccine. Blood samples were obtained before the first and second immunizations and 30 days after the second dose. In the longer schedule, 115 children received two doses 77 days apart. With lot 1, 30 children received undiluted vaccine and 25 children diluted vaccine; with lot 2, 30 children each received undiluted and diluted vaccine. Blood samples were obtained before the first and second doses and 18 days after the second dose. For each vaccine dose, 0.5 mL of freshly reconstituted or diluted vaccine was given subcutaneously in the upper arm, as indicated by the manufacturer.

Blood samples were allowed to clot. Serum samples were separated and stored frozen, initially at −20°C and later at −70°C.

Neutralization tests. Serum samples were inactivated at 56°C for 0.5 h. Serial dilutions (2-fold) were made from an initial 1:2.5

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Consent was obtained from subjects' parents.
Reprints or correspondence: Dr. Theodore F. Tsai, Division of Vector-Borne Infectious Diseases, CDC Foothills Campus, P.O. Box 2087, Fort Collins, CO.

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dilution. About 100 pfu of JE virus (Nakayama strain) was incubated with each serum dilution overnight at 4°C. The virus-serum mixtures (final serum dilution of 1:5) were inoculated onto drained Vero cell monolayers grown in 6-well panels. After viral adsorption (0.5 h at 37°C), monolayers were overlaid with a solid nutrient medium. One week later, a second layer containing neutral red was added; plaques were counted 24–48 h later. The 50% plaque reduction end-point titer was calculated by probit analysis; a titer of 1:10 was considered positive [6].

Results

No prevaccination serum sample contained JE neutralizing antibody. Responses to diluted and undiluted vaccine were similar, and results for the subgroups were combined. After the first dose, 72%–100% of vaccinees responded (figure 1). When a second dose was given 1–3 months later, 94%–100% of vaccinees developed an antibody response. The longer immunization schedule did not increase the proportion of responders; however, geometric mean antibody titers (GMTs) were higher in subjects vaccinated over a 3-month interval. In the short schedule, GMTs rose from 46 to 89 for lot 1 and from 39 to 65 for lot 2. With the long schedule, GMTs rose from 25 to 158 for lot 1 and from 19 to 115 for lot 2.

Lot 1 produced a significantly higher response rate 30 days after the first dose (100% vs. 72% for lot 2, $P < .001$), and there was a trend toward a higher response rate 18 days after the second dose was given in the long schedule (100% vs. 94%, $P = .11$).

Discussion

JE virus is the leading childhood viral central nervous system infection in Asia, with an annual incidence of 50,000 cases, >10-fold that of acute flaccid paralysis due to poliomyelitis. In areas where the disease is endemic, its public health burden is significant. Disease incidence rates are in the range of 5/100,000 persons, 5%–30% of cases are fatal, and one-third of survivors have serious residual neurologic deficits. In endemic areas, about one-half of all JE cases occur in children <4 years of age and nearly all cases occur in children <10; thus, early protection in childhood is needed [1–3].

The only internationally distributed JE vaccine is an inactivated vaccine purified from infected mouse brains. Although it is efficacious, the vaccine is troubled by uncertain rates of adverse reactions in recipients, and its mouse brain substrate is a limiting factor in acceptability and production cost. Pre- and postlicensure studies in the United States have disclosed hypersensitivity reactions consisting of generalized urticaria and angioedema in 0.5% of vaccinees, a rate that has been considered acceptable for selected military personnel and travelers at high risk of infection but not for travelers at large [7, 8].

Compared with other routinely used vaccines, the mouse brain–derived vaccine's reactogenicity is considerably greater. Recent reports of temporally related, acute disseminated encephalomyelitis have refocused attention on the possibility of vaccine-related neurologic side effects. Published risk estimates are difficult to interpret, but a recent estimate ranged as high as 1/100,000 doses [9, 10]. Although the immunogen is highly purified from its mouse brain substrate, numerous unidentified protein bands are seen on silver-stained electrophoretic gels, of which some could be of mouse origin. Finally, the complexity and cost of producing the vaccine is a significant impediment to large-scale production in developing Asian countries, where it is needed. It is doubtful that manufacturing capacity exists to fully immunize the Asian birth cohort of 70 million children.

A live attenuated JE vaccine grown in cell cultures, made from the SA14-14-2 strain, was developed in China and

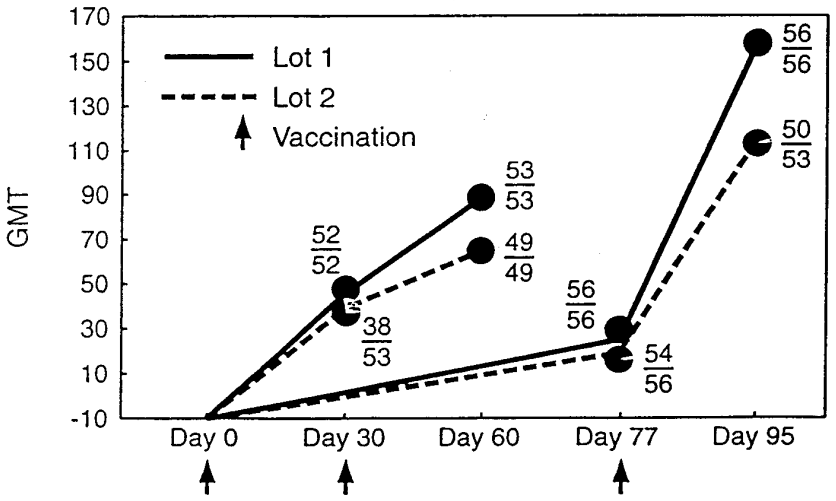

Figure 1. Neutralizing antibody responses determined by 50% plaque reduction neutralization tests of vaccinees given 2 doses of SA14-14-2 vaccine in 1- or 3-month interval and for 2 vaccine lots. The proportion of subjects with antibody titers >10 is shown for each point. Geometric mean antibody titers (GMTs) are given in text.

since 1988 has been safely used in >100 million children in southern China. No significant adverse events were seen in separate prospective studies of 867 and 13,275 children, and the vaccine's efficacy of >95% has been reconfirmed in an effectiveness study [4, 5, 11, 12]. JE vaccine currently is given in annual spring campaigns to children >1 year of age rather than at a specific chronologic age. The vaccine is produced each winter and is made available to local health clinics during a limited interval of several months. If a child misses that annual opportunity, he or she must wait until the next year to be immunized. Under this system, compliance with vaccination is difficult to follow and vaccine coverage undoubtedly is compromised. Systematic JE vaccination at a specific chronologic age in conjunction with other routine childhood immunizations would improve vaccine coverage and also could reduce vaccine administration costs.

The results of our study showed that two doses of SA 14-14-2 vaccine given either 1 or 2.5 months apart confers immunity in nearly all vaccinees. Neutralizing antibody titers were higher in vaccinees receiving two doses separated by 2.5 months despite the shorter postimmunization blood-sampling interval of 18 days (vs. 30 days after the 1 month schedule). It is likely that higher antibody titers would have been detected in a later blood sample. However, antibodies in children immunized with the 1-month schedule also were at levels considered to be protective. It is unknown whether antibody persistence in the 2 groups will differ. Antibody responses to undiluted vaccine and to a 3-fold dilution were similar, supporting previous observations that the minimal infectious titer of an immunizing dose is approximately $\log_{10}$ 6 pfu/mL [1].

The principal limitation of the study is the age of subjects, who were children 12–15 years old. Chinese authorities required the initial evaluation of the experimental schedule to be conducted in older children. Further studies are needed to confirm immune responses to these schedules in 9- to 18-month-old infants, the principal target group for primary immunization. In most Asian countries, the mouse brain–derived JE vaccine is given in two doses no earlier than age 12 months, with frequent boosters at 1- to 3-year intervals thereafter. The first dose of SA14-14-2 vaccine currently is given to infants ~9–18 months old and the second dose is given ~1 year later. Occasionally, younger infants are immunized inadvertently.

It would be desirable to administer JE vaccine simultaneously with other childhood vaccines in the EPI schedule. It probably is imprudent to give the mouse brain–derived JE vaccine with diphtheria–tetanus toxoid–pertussis (DTP) vaccine containing adjuvant because of the potential for allergic encephalomyelitis. Furthermore JE cases rarely occur before 2 years of age [13]. Although there is a potential for interference from maternal immunity, SA14-14-2 vaccine could be given at the last scheduled EPI visits for DTP and measles vaccination at age 6–12 months. Preliminary data indicate that only high levels of JE neutralizing antibody (>1:80) reduce the humoral response to SA14-14-2 vaccine, suggesting that waning maternally derived antibody may not interfere with the immune response in infants of that age [14]. Studies of combined JE and measles vaccination in infants are planned in China, using an experimental combined vaccine (Yu YX, unpublished data).

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INCLEN Clinical Epidemiology Unit, and Department of Pediatrics, West China University of Medical Sciences, and Chengdu Anti-epidemic Station, Chengdu, Sichuan, China; Department of Biostatistics and Epidemiology, Center for Clinical Epidemiology and Biostatistics, and Division of General Internal Medicine, Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania; Centers for Disease Control and Prevention, Fort Collins, Colorado; National Naval Medical Center, Bethesda, Maryland

The short-term safety of an effective and inexpensive new live attenuated Japanese encephalitis vaccine (SA14-14-2) was studied in a randomized trial, using block randomization. Of 26,239 children who were enrolled, half received the vaccine and half served as controls. Subjects were prospectively followed for 30 days for severe adverse events, such as encephalitis, meningitis, and "all-cause" hospitalization. No cases of encephalitis or meningitis occurred in either group. The upper 95% confidence limit for adverse events not occurring among subjects receiving their first dose was 4.1/10,000. Risk ratios and 95% confidence intervals for other adverse events were 0.70 (0.43–1.15) for all-cause hospitalization, 0.91 (0.37–2.22) for seizure, and 0.79 (0.56–1.11) for fever lasting ≥3 days. These data attest to the short-term safety of the SA14-14-2 virus strain and the hamster kidney cell substrate.

Japanese encephalitis (JE) is an important public health problem throughout a vast region of Asia. Conservative estimates place the annual incidence at >35,000 cases, mostly in children [1]. Among these, >10,000 die of JE, and an equal number develop permanent neurologic sequelae. JE is caused by a flavivirus that circulates in zoonotic cycles involving many vertebrate species and is transmitted to humans by the bite of several mosquito species. Because of its zoonotic cycle, prospects for eradicating JE from the environment are dim, and universal childhood vaccination is likely to remain essential for its control in the foreseeable future.

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In 1988, an inexpensive (US $.75/dose) live attenuated primary hamster kidney–derived JE vaccine (SA14-14-2) was licensed in China [4]. Prior to licensure, trials conducted in highly endemic areas indicated 95% efficacy after a single dose [1, 5]. A recent case-control study conducted in an area less endemic for JE showed 80% effectiveness (95% CI, 44%–93%) after one dose and 97.5% effectiveness (95% CI, 86%–99.6%) after two doses administered 1 year apart [6].

Some information concerning the safety of this vaccine is known. Results of a nonexperimental cohort study of the candidate vaccine were published in the Chinese literature [5]. Although the study lacked methodologic detail, it showed that no serious events were detected among 588,512 vaccinees. The vaccine's safety was also studied among 1026 children (5–12 years old) who were followed for 14 days after receiving their first dose of this vaccine. No cases of encephalitis or other serious adverse events were observed [4]. The present trial was done to complement earlier data with a more formal study of the 30-day safety of the SA14-14-2 virus strain and of hamster kidney cells as a substrate for the production of live attenuated vaccines.

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Reprints or correspondence: Dr. Sean Hennessy, 832 Blockley Hall, University of Pennsylvania, Philadelphia, PA 19104-6021.

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Methods

Study design. The randomized trial used health centers as the unit of randomization. Subjects were assigned to receive or not receive vaccine and were prospectively followed for 30 days for serious adverse events, such as encephalitis, meningitis, and "all-cause" hospitalization. In addition, a convenience subsample of vaccinated subjects was followed more closely for 7 days in order to identify mild adverse events.

Setting. The study was done in Chengdu, the capital of Sichuan Province, People's Republic of China. Children in Chengdu are vaccinated with JE vaccine at approximately ages 1, 2, and 6 years during campaigns occurring each spring. In response to announcements regarding vaccination campaigns for a particular year, children are brought to neighborhood health centers, which provide primary health care, including vaccinations. No other vaccine is administered concurrently.

Study population. Study subjects included all children who presented for JE immunization to one of the participating health centers during the 1995 vaccination campaign and who possessed a vaccination card indicating no or one previous dose of JE vaccine. Specific health centers were selected for enrollment on the basis of the number of children served and the willingness of center staff to participate. Health center workers were trained and given written instructions on study procedures. A sufficient number of centers was recruited to enroll at least 10,000 exposed and 10,000 unexposed children so that adverse events as rare as 3/10,000 could be detected [7].

Randomization. Participating health centers were randomized to either treatment or control status in the following manner. First, centers were pair-matched within each of the five urban districts (based on the neighborhood and the number of children served by each center). Smaller centers were combined with two or three others for purposes of randomization. Then, for each pair, a drawing was done to randomly assign one center to treatment status and the other to control status.

Interventions. Children presenting to either type of health center underwent an initial evaluation that consisted of an examination of their vaccination card and a structured interview with their parent or caretaker. Children presenting to treatment centers were then vaccinated at this initial visit, while children presenting to control centers had their vaccination delayed until after the observation period. The vaccine was the same one that is routinely used in those health centers, and it was manufactured, as described elsewhere [1], by the Chengdu Biological Products Institute (Chengdu).

Outcome measures. All illnesses prompting a health center visit during the study period were recorded, including the pediatrician's diagnosis. In addition, subjects and parents from both groups were asked to return to the center 30 days after the enrollment visit. Parents then underwent a structured interview regarding hospitalizations and illnesses that occurred since the initial visit. Children in the control group were then vaccinated. In an intensive effort to attain complete follow-up, study personnel made home visits to subjects who did not return after 30 days.

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Medical records for all hospitalized subjects were examined by abstractors who were blinded to the study group. Because the primary outcomes of interest (encephalitis and meningitis) are severe events that should reliably result in hospitalization, these outcomes were defined on the basis of a physician diagnosis. Bronchitis was defined on the basis physician diagnosis from a hospital admission or health center visit. Other outcomes (severe reactions consistent with anaphylaxis, seizure, fever lasting $\geq$3 days, diarrhea, and upper respiratory infection) were diagnosed on the basis of a parent report or a physician diagnosis.

A convenience sample of 266 vaccinated subjects was enrolled for a more intensive evaluation consisting of a brief physical examination performed by a study pediatrician at days 1, 2, 3, and 7 after vaccination.

Statistical analysis. Risk ratios and 95% CIs that accounted for clustering by health center were calculated by use of the CSAMPLE component of Epi Info (version 6.04; Centers for Disease Control and Prevention, Atlanta) [8]. Epi Info was also used to calculate exact mid-P 95% CIs [9] for the incidence of adverse events among vaccinees.

Results

Of the 180 participating health centers, 104 were assigned treatment status and 76 were assigned control status. There were 26,239 eligible subjects, with 13,275 of these presenting to treatment centers and 12,964 presenting to control centers. The groups were well-balanced with respect to prevaccination factors: The mean (SD) age was 1.9 (0.6) years, 52% of subjects were boys, and 55% were due to receive their first JE vaccination.

The mean (SD) duration of follow-up in the vaccinated group was 30.1 (1.5) days, and in the unvaccinated group, it was 30.1 (1.2) days. Loss to follow-up occurred in only 9 exposed subjects and 12 unexposed subjects, for a total of 21 losses to follow-up (8/10,000). Thirteen of these subjects were known to have moved out of Chengdu and could not be located. There was one death, which was due to an automobile accident that occurred in an unvaccinated subject.

The frequency of adverse events observed within 30 days of vaccination is presented in table 1. None of the events of primary interest (encephalitis, meningitis, or all-cause hospital admission) occurred more frequently in the vaccinated group than in the unvaccinated group. These results did not differ when subgroups were analyzed on the basis of whether the

Table 1. Number (%) of subjects with complete follow-up who experienced adverse events in the 30 days following immunization with JE vaccine.

Event	Vaccinated group (n = 13,266)	Unvaccinated group (n = 12,951)	Risk ratio (95% confidence interval)*
Encephalitis	0 (0.0)	0 (0.0)	Undefined
Meningitis	0 (0.0)	0 (0.0)	Undefined
Hospital admission	82 (0.6)	114 (0.9)	0.70 (0.43–1.15)
Severe reaction consistent with anaphylaxis	0 (0.0)	0 (0.0)	Undefined
Seizure	14 (0.1)	15 (0.1)	0.91 (0.37–2.22)
Fever lasting ≥3 days	357 (2.7)	442 (3.4)	0.79 (0.56–1.11)
Diarrhea	12 (0.1)	11 (0.1)	1.06 (0.46–2.49)
Upper respiratory infection	292 (2.2)	353 (2.7)	0.81 (0.55–1.18)
Bronchitis	38 (0.3)	44 (0.3)	0.84 (0.49–1.44)

* Accounts for clustering by health center [8].

vaccine administered was the first or second dose (data not shown). No cases of encephalitis, meningitis, or severe systemic reaction consistent with anaphylaxis were observed in either group. Therefore, the point estimate for the incidence of each of these adverse events (and other event types that did not occur) among vaccinees was 0%. The upper 95% confidence limit (CL) for the incidence of these events in the vaccinated group overall (i.e., children receiving the first or second dose; n = 13,266) was 2.3/10,000. Among children receiving their first dose (n = 7262), the upper 95% CL was 4.1/10,000. Among children receiving their second dose (n = 6,004), the upper 95% CL was 5/10,000.

Evidence of adverse events was also sought by means of physical examination in 266 vaccinated subjects at days 1, 2, 3, and 7 after immunization. Similar to the case with the overall study population, the mean (SD) age in this subgroup was 1.9 (0.6) years, and 53% were boys. The adverse event data from this subgroup are presented in table 2. Fever was the most common adverse event, occurring in 13 subjects (4.9%), which was consistent with the incidence of prolonged fever in the overall vaccinated population (2.7%). Irritability was the second most common event, occurring in 10 subjects (3.7%). One subject was hospitalized with a diagnosis of pneumonia. No other serious events were observed in this group.

Discussion

This study provides convincing evidence of the 30-day safety of a live attenuated JE vaccine. In this large randomized trial, the incidence of adverse events in the exposed group was no higher than that of a concurrent control group. Further, because no cases of encephalitis, meningitis, or anaphylaxis occurred, the point estimate for the incidence of each of these events was 0%, with an upper 95% CL for the pooled incidence among first- and second-dose recipients of 2.3/10,000. Because children receiving their second dose might be at a lower risk of vaccine-induced JE (because of immunity acquired from the first dose), the incidence of encephalitis among children receiving their first dose is a more conservative measure of the vaccine's safety. The point estimate of the incidence of encephalitis in this group is also 0%, with an upper 95% CL of 4.1/10,000.

These results need to be interpreted in light of concerns common to all epidemiologic studies. Selection bias and confounding have been largely avoided because of random assignment, albeit by block randomization. Because hospitalization is a highly memorable event for the parents of a young child, we believe that underascertainment of outcomes based on parents' failure of recall is unlikely. This belief is strengthened by the observation that 98% of all hospital admissions detected through the surveillance system were also reported by parents. Further, if underascertainment did play a role, we believe that in this unblinded study, parents would be more likely to remember adverse events that occurred in vaccinated subjects than those that occurred in unvaccinated subjects, which would bias the study results against the vaccine.

This candidate live attenuated JE vaccine is known to be effective [1, 6], and previous evidence attesting to its short-term safety [4, 5] has now been confirmed in this large randomized trial. Thus, the short-term safety of both the SA14-14-2 virus strain and of hamster kidney cells as a substrate for the manufacture of live attenuated vaccines has been well-documented.

In summary, we have conducted a randomized trial in 26,239 children to evaluate the short-term safety of a live attenuated JE vaccine. The results indicate that the vaccine is not associated with an increased incidence of encephalitis, meningitis,

Table 2. Adverse events observed in a convenience sample of 266 vaccinated subjects examined at days 1, 2, 3, and 7 after JE immunization.

Event	No. of subjects experiencing event (n = 266)	% (95% confidence interval)
Fever ≥37.5°C	13	4.9 (2.7–8.2)
Hives	1	0.4 (0.02–1.8)
Angioedema	0	0 (0–1.1)
Joint swelling	0	0 (0–1.1)
Rash	6	2.2 (0.9–4.6)
Asthma	0	0 (0–1.1)
Cough	9	3.4 (1.6–6.1)
Injection-site tenderness	1	0.4 (0.009–2.1)
Axillary adenopathy	0	0 (0–1.1)
Irritability	10	3.8 (1.9–6.6)
Vomiting	3	1.1 (0.3–3.0)
Diarrhea	2	0.8 (0.12–2.5)
Seizure	0	0 (0–1.1)

all-cause hospitalization, or other serious adverse events in the 30 days following vaccination. This provides reassuring evidence of the short-term safety of an affordable vaccine with known effectiveness.

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Statistical analysis. Risk ratios and 95% CIs that accounted for clustering by health center were calculated by use of the CSAMPLE component of Epi Info (version 6.04; Centers for Disease Control and Prevention, Atlanta) [8]. Epi Info was also used to calculate exact mid-P 95% CIs [9] for the incidence of adverse events among vaccinees.

Results

Of the 180 participating health centers, 104 were assigned treatment status and 76 were assigned control status. There were 26,239 eligible subjects, with 13,275 of these presenting to treatment centers and 12,964 presenting to control centers. The groups were well-balanced with respect to prevaccination factors: The mean (SD) age was 1.9 (0.6) years, 52% of subjects were boys, and 55% were due to receive their first JE vaccination.

The mean (SD) duration of follow-up in the vaccinated group was 30.1 (1.5) days, and in the unvaccinated group, it was 30.1 (1.2) days. Loss to follow-up occurred in only 9 exposed subjects and 12 unexposed subjects, for a total of 21 losses to follow-up (8/10,000). Thirteen of these subjects were known to have moved out of Chengdu and could not be located. There was one death, which was due to an automobile accident that occurred in an unvaccinated subject.

The frequency of adverse events observed within 30 days of vaccination is presented in table 1. None of the events of primary interest (encephalitis, meningitis, or all-cause hospital admission) occurred more frequently in the vaccinated group than in the unvaccinated group. These results did not differ when subgroups were analyzed on the basis of whether the

Table 1. Number (%) of subjects with complete follow-up who experienced adverse events in the 30 days following immunization with JE vaccine.

Event	Vaccinated group (n = 13,266)	Unvaccinated group (n = 12,951)	Risk ratio (95% confidence interval)*
Encephalitis	0 (0.0)	0 (0.0)	Undefined
Meningitis	0 (0.0)	0 (0.0)	Undefined
Hospital admission	82 (0.6)	114 (0.9)	0.70 (0.43–1.15)
Severe reaction consistent with anaphylaxis	0 (0.0)	0 (0.0)	Undefined
Seizure	14 (0.1)	15 (0.1)	0.91 (0.37–2.22)
Fever lasting ≥3 days	357 (2.7)	442 (3.4)	0.79 (0.56–1.11)
Diarrhea	12 (0.1)	11 (0.1)	1.06 (0.46–2.49)
Upper respiratory infection	292 (2.2)	353 (2.7)	0.81 (0.55–1.18)
Bronchitis	38 (0.3)	44 (0.3)	0.84 (0.49–1.44)

* Accounts for clustering by health center [8].

vaccine administered was the first or second dose (data not shown). No cases of encephalitis, meningitis, or severe systemic reaction consistent with anaphylaxis were observed in either group. Therefore, the point estimate for the incidence of each of these adverse events (and other event types that did not occur) among vaccinees was 0%. The upper 95% confidence limit (CL) for the incidence of these events in the vaccinated group overall (i.e., children receiving the first or second dose; n = 13,266) was 2.3/10,000. Among children receiving their first dose (n = 7262), the upper 95% CL was 4.1/10,000. Among children receiving their second dose (n = 6,004), the upper 95% CL was 5/10,000.

Evidence of adverse events was also sought by means of physical examination in 266 vaccinated subjects at days 1, 2, 3, and 7 after immunization. Similar to the case with the overall study population, the mean (SD) age in this subgroup was 1.9 (0.6) years, and 53% were boys. The adverse event data from this subgroup are presented in table 2. Fever was the most common adverse event, occurring in 13 subjects (4.9%), which was consistent with the incidence of prolonged fever in the overall vaccinated population (2.7%). Irritability was the second most common event, occurring in 10 subjects (3.7%). One subject was hospitalized with a diagnosis of pneumonia. No other serious events were observed in this group.

Discussion

This study provides convincing evidence of the 30-day safety of a live attenuated JE vaccine. In this large randomized trial, the incidence of adverse events in the exposed group was no higher than that of a concurrent control group. Further, because no cases of encephalitis, meningitis, or anaphylaxis occurred, the point estimate for the incidence of each of these events was 0%, with an upper 95% CL for the pooled incidence among first- and second-dose recipients of 2.3/10,000. Because children receiving their second dose might be at a lower risk of vaccine-induced JE (because of immunity acquired from the first dose), the incidence of encephalitis among children receiving their first dose is a more conservative measure of the vaccine's safety. The point estimate of the incidence of encephalitis in this group is also 0%, with an upper 95% CL of 4.1/10,000.

These results need to be interpreted in light of concerns common to all epidemiologic studies. Selection bias and confounding have been largely avoided because of random assignment, albeit by block randomization. Because hospitalization is a highly memorable event for the parents of a young child, we believe that underascertainment of outcomes based on parents' failure of recall is unlikely. This belief is strengthened by the observation that 98% of all hospital admissions detected through the surveillance system were also reported by parents. Further, if underascertainment did play a role, we believe that in this unblinded study, parents would be more likely to remember adverse events that occurred in vaccinated subjects than those that occurred in unvaccinated subjects, which would bias the study results against the vaccine.

This candidate live attenuated JE vaccine is known to be effective [1, 6], and previous evidence attesting to its short-term safety [4, 5] has now been confirmed in this large randomized trial. Thus, the short-term safety of both the SA14-14-2 virus strain and of hamster kidney cells as a substrate for the manufacture of live attenuated vaccines has been well-documented.

In summary, we have conducted a randomized trial in 26,239 children to evaluate the short-term safety of a live attenuated JE vaccine. The results indicate that the vaccine is not associated with an increased incidence of encephalitis, meningitis,

Table 2. Adverse events observed in a convenience sample of 266 vaccinated subjects examined at days 1, 2, 3, and 7 after JE immunization.

Event	No. of subjects experiencing event (n = 266)	% (95% confidence interval)
Fever ≥37.5°C	13	4.9 (2.7–8.2)
Hives	1	0.4 (0.02–1.8)
Angioedema	0	0 (0–1.1)
Joint swelling	0	0 (0–1.1)
Rash	6	2.2 (0.9–4.6)
Asthma	0	0 (0–1.1)
Cough	9	3.4 (1.6–6.1)
Injection-site tenderness	1	0.4 (0.009–2.1)
Axillary adenopathy	0	0 (0–1.1)
Irritability	10	3.8 (1.9–6.6)
Vomiting	3	1.1 (0.3–3.0)
Diarrhea	2	0.8 (0.12–2.5)
Seizure	0	0 (0–1.1)

all-cause hospitalization, or other serious adverse events in the 30 days following vaccination. This provides reassuring evidence of the short-term safety of an affordable vaccine with known effectiveness.

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PROTECTIVE EFFICACY OF SA14-14-2 ATTENUATED JAPANESE ENCEPHALITIS VACCINE, CHINA

Province	Year	Study Group	Number of Subjects	JE Cases	Incidence /100,000	Efficacy (95% CI)
Guizhou	1988	Vaccinated*	86,132	1	1.16	98.0 (96-100)
		Unvaccinated	21,149	12	56.7	
	1989	Vaccinated*	86,933	0	2.30	100
Jiang-Xi	1989	Unvaccinated	16,869	12	71.1	
		Vaccinated*	64,027	2	3.12	98.4 (97-100)
		Unvaccinated	4546	9	198.0	
	1990	Vaccinated+	63,927	1	1.56	99.8 (98-100)
		Unvaccinated	5,784	37	639.6	
	1991-1993	Vaccinated+	~65,000	0		100
		Unvaccinated	~7,000	23 (3years)	~109.6	
Yunnan	1991	Vaccinated#	29,639	2	6.75	95.7 (94-99)
		Unvaccinated	29,006	46	158.6	
Anhui	1992	Vaccinated	145,758	2	1.37	99.3 (99-100)
		Unvaccinated	11,264	22	195.3	

* Children 1-10 years immunized with single primary dose and booster 1 year later.
+ Second or more years of observation.
# Children 1-7 years lod immunized with single primary dose only.

Primary and booster immune responses to SA14-14-2 Japanese encephalitis vaccine in Korean infants

Young Mo Sohn M.D.[1]

Min Soo Park M.D.[1]

Hye Ok Rho M.D.[1]

Laura Chandler PhD.[2]

Robert Shope M.D.[2]

Theodore F. Tsai M.D.[3*]

[1]Dept. of Pediatrics

Yonsei University College of Medicine

Seoul, Korea

[2]Dept. of Pathology

University of Texas Medical Branch

301 University Blvd.

Galveston, TX 77555-0609

[3]Division of Vectorborne Infectious Diseases

CDC

Ft. Collins, CO 80522

***corresponding author**

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Abstract

Attenuated SA14-14-2 Japanese encephalitis (JE) vaccine has been administered safely and effectively to more than 100 million children in China since 1988 and recently, licensure of the vaccine in Korea has been sought. In the first clinical evaluation of the vaccine outside of China, we monitored side effects in 84 children and evaluated antibody responses to a single dose given as primary JE vaccination in 68 children, 1-3 years old (mean age 27 months). No significant adverse events were noted. Neutralizing antibodies (geometric mean titer [GMT] of 188) were produced in 96% of the 68 subjects. In 10 other children who previously had been immunized with two or three doses of inactivated JE vaccine, the booster administration of SA14-14-2 vaccine produced an anamestic response in all, with a GMT of 3378. In a comparison group of 25 children previously immunized with two doses of inactivated vaccine, neutralizing antibody titers were detected in 16 (64%). Viral specific IgM was detected in nine primary vaccinees (13%) but in others, IgM may have declined to undetectable levels in the four week postimmunization sample. Live attenuated SA14-14-2 JE vaccine is a promising alternative to the only commercially available JE vaccine for national childhood immunization programs in Asia.

Introduction

Japanese encephalitis (JE) is the leading recognized cause of childhood viral encephalitis in Asia. Over 50,000 cases, with a case-fatality ratio of 5-35%, are estimated to occur annually in the region (1,2). National vaccination programs in Japan, Taiwan and in Korea, using an inactivated mouse-brain derived vaccine, have controlled the disease to the point of elimination but in other countries, the expense and complexity of producing the vaccine and the need for numerous doses have limited vaccine implementation. Typically, two to three doses are given in the primary immunization series, followed by biennial or even annual boosters through childhood to maintain immunity, for a total of 6-12 doses (2). In addition to the limitations posed by multiple doses, as the vaccine has been introduced elsewhere to protect travelers, a high rate of hypersensitivity events has been reported among vaccine recipients in North America, Europe and Australia (2-4).

A live-attenuated JE vaccine made from the SA14-14-2 strain was developed in China and since its licensure in 1988, has been given safely to more than 100 million children (2). The vaccine is given annually, in spring campaigns, to infants over one year and is 98% effective when two doses are administered (5). Recently, a New Drug Application (NDA) for the vaccine was submitted in Korea. We report here the first clinical experience with SA14-14-2 vaccine outside of China and confirm that in Korean infants a single dose of the attenuated vaccine was highly immunogenic. In a subset of infants who previously had received primary

or primary plus booster immunization with inactivated JE vaccine, we show that the attenuated vaccine produced an anamnestic response.

Materials and Methods

Study locations and subjects.

The study protocol was approved by Institutional Review Boards of Yongdong Severance Hospital, Yonsei University College of Medicine and the Central Pharmaceutical Affairs Council, National Drug Committee in the Korea Ministry of Health and Welfare. We sought study subjects between one and three years of age from three private chronic care facilities located in areas where JE prevalence is low- two in Seoul (50 and 20 children, respectively) and one in Kyungki Province (41 children) for a total of 111 children. Informed consent for each subject was obtained from the guardian. We reviewed their immunization records, performed full physical examinations, clinical laboratory screening tests including complete blood cell counts, platelets, alanine amino transferase (ALT), aspartate aminotransferase (AST) and a preimmunization JE viral antibody determination, using a plaque reduction neutralization test (PRNT). Immunization records at private chronic care facilities were examined to validate vaccination histories. After physical examination, 8 children were excluded from the study because they had chronic medical illnesses and 17 because they were over three years of age and previously had been immunized with inactivated JE vaccine. A blood sample was obtained to measure their current JE PRNT antibody titer.

The remaining 86 subjects were due for JE vaccination, as recommended by national vaccination policy, and were immunized with one dose of SA14-14-2 vaccine. However, 10 later were discovered to have been immunized with inactivated JE vaccine, of whom six had JE PRNT antibodies in the preimmunization sample and four did not. In addition, six other children without a record of JE vaccination had JE PRNT antibody titers in the preimmunization blood sample, for a total of 16 subjects who either had a history of previous JE immunization or infection. Four weeks after immunization, two subjects were lost to follow up, leaving a total of 68 subjects who could be evaluated for their primary immune response to SA14-14-2 vaccine. Their mean age was 26.9 ± 6.6 months and 39 were boys (Table 1). In the 10 subjects who had a history of immunization with inactivated JE vaccine (with or without measurable PRNT antibodies) and in the six children with naturally acquired JE antibody, the single dose of SA14-14-2 vaccine was, in effect, a booster vaccination.

In the 10 previously immunized study subjects and in the 17 other children who were over the three years of age for inclusion in the study (see above), 25 had received two doses of mouse brain-derived JE vaccine (Nakayama strain) in the usual primary series and two had received an additional booster dose. We measured post-immunization neutralizing antibody responses to the inactivated vaccine in all 27 to compare their postimmunization PRNT antibody titers with those in the 68 immunized with attenuated vaccine.

Vaccination and bleeding schedules

SA14-14-2 vaccine was produced by the Chengdu Institute of Biological Products,

Chengdu, China. The vaccine lot, 971050, was manufactured on 10/4/97 and was used before the expiration date of 4/3/99. The lot had an infectious titer of 6.8 $\log_{10}$ pfu/0.5ml, titrated by the Chengdu Institute of Biological Products and confirmed at the National Institute for Control of Pharmaceutical and Biological Products, Ministry of Public Health, Temple of Heaven, Beijing. Single doses of lyophilized vaccine were reconstituted with water for injection supplied and as prescribed by the manufacturer. Reconstituted vaccine was used immediately.

For each subject, 0.5 mL of freshly reconstituted vaccine was given subcutaneously in the upper arm, as indicated by the manufacturer. Venous blood samples were obtained at the first visit, prior to immunization, and again four weeks after immunization. Blood samples (5 mL each) were divided into 2 aliquots. One was placed in an EDTA tube for complete blood and platelet counts and the other in a plain tube. Blood in the plain tube was allowed to clot and serum was separated by centrifugation and divided into 2 aliquots: one for blood chemistry (AST and ALT) determinations and the other further dispensed into 0.5mL aliquots and stored frozen in a number of 1.0 mL flat-bottom Nunc cryovials at -70C for serological tests. The cryovials were labeled with code numbers only. The serum samples for antibody determinations were sent frozen on dry ice to the WHO Collaborating Center for Tropical Diseases, Arbovirus Referenoe Center, University of Texas Medical Branch (UTMB), Galveston, TX. The safe arrival of the samples within 24 hours was confirmed.

In previously immunized children, an examination of clinic records disclosed that

inactivated mouse brain derived (Nakayama strain) JE vaccine from various Korean distributors and various lots had been administered according to routine practices. Primary immunization consisted of two doses given approximately two weeks apart. Blood samples for PRNT determinations were obtained approximately seven months after completion of either the primary or booster immunization.

Adverse events

After vaccination, all subjects were observed and monitored for at least 30 minutes by a physician or study nurse for any signs or symptoms of local and/or systemic reaction. Subjects also were asked to report immediately the occurrence of any local or systemic symptoms during the month after immunization and, they were systematically examined at prevaccination screening, on the day of vaccination and 30 days after immunization to record local or systemic adverse reactions.

Serological determinations

All serological tests were performed at UTMB on coded samples.

Plaque reduction neutralization tests (6) Sera were heat inactivated at 56C for 0.5 h and diluted 1:5 and in serial twofold dilutions. The SA14 strain of JE virus was diluted in PBS containing 5%FCS and 5% guinea pig complement to provide 200 pfu/0.1 ml, and added in equal volume to each serum dilution. Serum-virus mixtures were incubated overnight at 4C and added to drained Vero cell culture monolayers grown in six well plates. After adsorption for one hour at 37C, the monolayers were overlaid with semisolid medium. Approximately seven days later, when plaques could be seen microscopically, the medium was removed, the

monolayer was fixed with 20% methanol and stained with crystal violet. The complete titration of each serum was carried out in a single test (final serum dilutions of 1:10 to 1:5120). Endpoint titers were calculated as highest last dilution that inhibited 50% of the viral input dose.

IgM capture ELISA (7) Diluted serum samples (1:100) were added to 96 well plates coated with antihuman-μ chain antibody. JE viral (Nakayama strain) or control inactivated, sucrose acetone extracted-mouse brain antigen was added, followed by enzyme-labeled antiflaviviral monoclonal antibody and substrate. Absorbance values of viral and control wells were measured and samples with a positive/negative ratio >2 were considered positive.

Results

Adverse events

No immediate local or systemic reactions were observed. During the one month interval after immunization, the following mild and self-limited symptoms and signs were reported: elevated temperature (37.5° – 38° C) 7% (6/86); vomiting 1% (1/86); skin rash 1% (1/86); loss of appetite 1% (1/86); and irritability 1% (1/86). One child developed an upper respiratory illness with thrombocytopenia four weeks after vaccination. The platelet count declined from 148,000/mm^3, in the prevaccination sample, to 53,000/mm^3 but returned to normal within one month. There were no significant changes in results of CBC, ALT and AST determinations between prevaccination and postvaccination samples, but platelet counts

declined significantly, from 345,333 ± 86,919/mm^3 to 295,416 ± 78,032/mm^3, although they remained within the normal range.

Neutralizing antibody response to primary immunization with attenuated vaccine

Among the 68 children in whom vaccination records and absent preimmunization antibody indicated no prior exposure to JE virus or JE vaccine, the single primary immunizing dose of SA14-14-2 vaccine produced neutralizing antibodies in 65 (96%) (Table 1). The geometric mean titer (GMT) among the 65 reactors in this group of primary vaccinees was 188.

Neutralizing antibody response to booster immunization with attenuated vaccine

The 16 children, who had either a history of previous vaccination with inactivated vaccine and/or detectable JE viral PRNT antibodies in the preimmunization sample, all seroconverted after a single dose of SA14-14-2 vaccine (Table 1). The GMT in the 16 subjects was 1733. The booster immune response in the 10 children with a documented history of immunization with inactivated JE vaccine (GMT 3378) was more than 18-fold higher than the primary immune response in the 65 children reported above and responses were similar, irrespective of detectable PRNT antibodies in the prebooster sample.

Six children who had been immunized with inactivated JE vaccine and who had measurable prebooster PRNT antibodies, developed a >100-fold rise in PRNT antibody after the booster.

Neutralizing antibody titers after primary immunization with inactivated vaccine

After primary immunization with two doses of inactivated JE vaccine, 16 of 25 children (64%) had measurable PRNT antibodies; the GMT of the seropositive samples was 104. One of two children who had received three doses of inactivated vaccine (two primary and a booster dose) had a PRNT antibody titer of 1:40, the other was seronegative.

IgM antibody responses

JE IgM antibodies were detected in the postimmunization serum sample of nine of 68 children (13%) who received primary immunization with SA14-14-2 vaccine but in none of the children with prior JE vaccination history or infection. The geometric mean neutralizing antibody titer of children in whom an IgM response was detected was lower than in those without detectable IgM (93 vs 205) but the difference was not significant ($p = 0.08$, t test).

Discussion

The attenuated SA14-14-2 JE virus strain, derived by serial passage in primary hamster kidney cells, was licensed and has been distributed in areas of southern China since 1988 (2). Neuroattenuation of the vaccine strain was demonstrated in experimental animals and in several large scale human studies, of which the most recent, a controlled study of 25,000 children, found no cases of central nervous system infection in 13,000 immunized children (8).

Prelicensure immunogenicity studies, in which the antibody response to one dose has been as low as 85% with geometric mean titers on the order of 23-46 have suggested that the vaccine strain may be overattenuated (2,9,10). Immune responses to vaccine produced after it's licensure in 1988 and containing ≥ 6.0 $\log_{10}$ pfu/ml have been more consistent. Although several field studies reported protective efficacies exceeding 95% after a single dose, based upon the vaccine's variable immunogenicity and exigencies of the traditional approach of campaign vaccination each spring, public health authorities have recommended two doses, given in successive years. This recommendation received support in a recent case-control effectiveness study that found an 80% vaccine effectiveness in children receiving one dose, and 98% after two doses (5).

The SA14-14-2 vaccine currently is distributed only in China but the attenuated vaccine has been recognized as a potentially less costly and less reactogenic alternative to the inactivated mouse brain-derived vaccine, which now is the only internationally distributed JE vaccine. In seeking licensure for SA14-14-2 vaccine in Korea, the manufacturer introduced certain changes in quality control procedures, which are reflected in the production of the launch lot used in this study. This preliminary immunogenicity study in Korean infants sought to reconfirm the vaccine's safety and immunogencity and was the first clinical evaluation of the vaccine outside of China.

We measured antibody responses in the primary immunization of children, one to three years old, the age interval when JE vaccine normally is given in Korea. Among the

children who received a single primary vaccinating dose, 96% developed a neutralizing antibody response, with a GMT 188 in responders. The proportion of seroconversions was similar to those in previous reports but the antibody titers produced in this series were higher, possibly reflecting methodological differences in the performance of PRNT and titer calculations (2,9,10). The post-immunization PRNT titer levels following SA14-14-2 vaccination are not dissimilar from those following vaccination with17D yellow fever (YF) vaccine, another attenuated flaviviral vaccines (11). From previous experience, a second dose of SA14-14-2 vaccine would be expected to produce tenfold higher neutralizing antibody titers (10).

In children who previously had been immunized with inactivated JE vaccine or who might have had a prior natural JE infection, SA14-14-2 vaccination produced an 18-fold PRNT antibody rise, consistent with an anamnestic response. This observation allays concern that children partially immunized with inactivated vaccine might have cleared the attenuated vaccine, before developing a secondary immune response. Equally high anamnestic antibody responses were produced in children who had no detectable neutralizing antibodies after previous vaccination with the inactivated vaccine, suggesting the retention of memory T cells, aiding the humoral response. Further studies to establish the duration of immunity after booster immunization with the SA14-14-2 vaccine and the need, if any, for additional boosters should be undertaken as the vaccine is introduced into routine immunization programs. In comparison to the 96% seroconversion among children immunized with the attenuated

vaccine, only 64% of children who had received two doses of inactivated vaccine had detectable PRNT antibodies. However, antibody responses in these children were not studied prospectively and the post-immunization samples were obtained, on average, seven months later, when antibody titers might have diminished. On the other hand, low seroconversion rates of 45-67% after two doses also have been in reported in studies from developed countries, leading to the current recommendation in the United States of three doses for primary vaccination (12,13). The anamnestic response to live attenuated SA14-14-2 vaccine in children who had received two doses of inactivated vaccine, however, supports observations from field studies that the two dose schedule is protective (14).

JE virus specific IgM antibodies were detected in only 13% of primary vaccinees, however, postimmunization serum samples were obtained four weeks after immunization and, in others, IgM antibodies might have declined to undetectable levels. For the same reason and because so few subjects were studied, although none of the 11 children with previous immunization or infection produced viral specific IgM, no conclusion can be drawn about the absence of IgM as evidence of a secondary antibody response. The trend toward higher neutralizing antibody titers among vaccinees in whom IgM was not detected suggests the possibility that some of these children may have been immunologically primed, and like those children with a history of previous vaccination without prevaccination antibodies, may have produced a secondary response to vaccination. Although this possibility cannot be excluded in individual cases, it is unlikely in any significant number of the group because of the low risk of

exposure to enzootic JE viral transmission in the locations where this study was undertaken.

The choice of the wild-type vaccine parent SA14 strain as the inoculum virus in PRNT determinations parallels the use of the parent Asibi strain in measuring YF viral antibody responses in YF 17D vaccine recipients, in whom it is well established that neutralizing antibody titers to wild-type YF virus consistently are lower than to the vaccine strain (11). By analogy, this observation suggests that we might have detected higher antibody titers had we used the SA14-14-2 strain itself as the challenge virus. Genomic sequence analysis has classified all JE vaccine strains, including SA14-14-2 and its SA14 parent, Nakayama, Beijing and P3 strains, in the genotype of JE viruses circulating in temperate areas of Asia but, no data indicate that these strains fail to protect against the disease occurring elsewhere in the region (15).

The excellent immune response to a single primary immunizing dose is encouraging and suggests that one dose may provide protective immunity. Although in a recent study, a single dose had an effectiveness of 80%, the observation was limited by the small study size, reflected in the wide confidence intervals of 40-93% around the estimate (5). The >95% protective efficacy of one dose in other field studies, seemingly at odds with lower immunogenicity rates, probably reflected the frequency of natural JE infections, of which >99% are asymptomatic, that primed or reinforced the response to vaccination (2).

In Korea and China, JE vaccine still is given in spring campaigns rather than in a schedule based on chronological age. Additional studies are needed to determine how the

SA14-14-2 vaccine should be integrated into pediatric immunization schedules in Asia.

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Figure Legends

Figure 1. Neutralizing antibody response to primary Japanese encephalitis (JE) vaccination with SA_{14}14-2 live attenuated JE vaccine. 50% plaque reduction neutralization test (PRNT) titers are shown for each of the 68 subjects before and four weeks after vaccination. The geometric mean titer and 95% confidence intervals of the post-vaccination samples was 185; 130-261. JE specific IgM antibodies were detected in nine vaccinees who had PRNTtiters of 10, 20, and 160 (nine vaccinees).

Figure 2 Anamnestic responses [50% plaque reduction neutralization test (PRNT) antibodies] to Japanese encephalitis (JE) vaccination using SA_{14}-14-2 live attenuated JE vaccine. The respective geometric mean PRNT titers with 95% confidence intervals were (open circles, n=4) children with history of vaccination with inactivated JE vaccine but no prevaccination PRNT antibody: pre-vaccination, <10 and post-vaccination 5120, 564-46,482; (closed circles, n=6) children with history of vaccination with inactivated JE vaccine and prevaccination PRNT antibodies: prevaccination 40, 14-112 and post-vaccination 7240, 2971-17,648; (diamonds, n=6) children with prevaccination JE antibodies and an uncertain vaccination history: pre-vaccination 45, 10-198 and post-vaccination 905, 96-8519; and for comparison, (squares, n=68) children receiving SA_{14}14-2 vaccine as a primary JE vaccination: pre-vaccination <10 and post-vaccination, 185, 130-261. Post-vaccination geometric mean PRNT titers were significantly higher in children previously immunized with inactivated JE vaccine than in primary vaccinees

($p<0.001$, t test).

Manufacturing outline
(Flow sheet)

Manufacturing Process Chart

Syrian primary hamster kidney cells

Incubation at 37 for 2 to 3 days with Cell Growth Media

Production Cell Culture
(Primary Hamster Kidney Cell Culture)

Observation for CPE

Test for Haemadsorbing viruses

Inoculation with working seed virus (2.7-3.7 logPFU/ml)

Incubation at 34-36℃ for 3-4 days with Maintenance media

Single Virus Harvest

Cooled down at 2-5℃

Clarification (filtration)

Bulk (Virus Pool)

Pooled and diluted

Final Bulk

Dispensed into final containers

Lyophilization below –20 °C

Final Vaccine

Quality control elements
(Outline of tests)

Quality Control

Working Virus Seed Stock

Sterility for bacteria, fungi

Mycoplasma

Virus Titration

Identity

Neurovirulence in monkeys

Attenuation test

- Mouse neurovirulence
- Mouse neuroinvasion
- Mouse reversion

Test for Adventitious viruses

Hamster colony

Periodic screening for hamster specific pathogens

Primary hamster kidney cell, Production and control cell culture

Observation of CPE

Haemadsorbing viruses

Non-haemadsorbing viruses

Other adventitious viruses

Single virus harvest

Sterility for bacteria, fungi

Mycoplasma

Mycobacterium Tuberculosis

Virus titer

Identity

Quality Control

Bulk (virus pool)

Sterility for bacteria, fungi

Mycoplasma

Mycobacterium Tuberculosis

Cell culture safety assay

In vivo safety assay

Virus contents

Final bulk

Sterility for bacteria, fungi

Mycoplasma

Attenuation test

- Mouse neurovirulence
- Mouse neuroinvasion
- Mouse reversion

Residual animal serum protein

Final vaccine

Identity

Virus titer

Thermostability

Sterility for bacteria, fungi

Abnormal toxicity

Moisture content

The quality control result of Japanese Encephalitis Vaccine, Live Attenuated

Item 项目 / No. 批号	980281	980282	980283	980284	980386	980387
1. Sterility Test 无菌试验	Negative	Negative	Negative	Negative	Negative	Negative
2. Test for residual Moisture content 水分	1.43	2.17	1.99	1.84	2.15	1.72
3. Test for virus Concentration (logPFU/ml) 病毒滴度	6.19	6.34	6.13	6.54	6.49	6.60
4. Thermostability Test（37℃） 热稳定性试验	5.74	5.35	6.02	5.72	6.0	5.94
5. Test for residual calf serum protein content(ng/ml) 残余牛血清蛋白含量	<3.125	<3.125	<3.125	<3.125	3.125	<3.125
6. Abnormal Toxicity Test 毒力试验	pass	pass	pass	pass	pass	pass
7. Intracerebral Inoculation of mice 脑内致病力	pass	pass	pass	pass	pass	pass
8. Subcutaneous Inoculation of mice 皮下感染入脑试验	pass	pass	pass	pass	pass	pass
9. Atavism Test 乳鼠传代返祖试验	pass	pass	pass	pass	pass	pass

1st division of viral vaccine

NICPBP

Date: 1999.3.2.