

# Japanese encephalitis virus live-attenuated vaccine, Chinese strain SA<sub>14</sub>-14-2; adaptation to primary canine kidney cell cultures and preparation of a vaccine for human use

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*The Japanese encephalitis (JE) live-attenuated vaccine virus clone SA<sub>14</sub>-14-2 was adapted to grow in primary canine kidney (PCK) cell culture, and vaccine seeds and a first lot of vaccine were prepared in these cells. Characterization of the PCK-grown virus by various laboratory and animal tests indicated that passage in PCK did not result in detectable phenotypic or genome changes for this virus clone. Markers of attenuation included small plaque size, lack of intracerebral virulence for weanling mice, minimal neurovirulence for rhesus monkeys and a distinct nucleotide pattern compared to the parent SA<sub>14</sub> non-attenuated virus. In addition, the seeds and vaccine were free of any detectable adventitious microbial agents that would render these materials unsafe for human immunization. Small-scale clinical trials of the JE SA<sub>14</sub>-14-2 PCK vaccine can now proceed to test the human safety of this product.*

## Introduction

Japanese encephalitis (JE) is a widespread viral disease in the People's Republic of China and, since 1975, 10 000–15 000 cases have been reported per year<sup>1</sup>. Due to the difficulty of controlling vectors and amplifying hosts, large amounts of efficacious JE vaccines are required for prevention of this disease in China.

A live-attenuated JE vaccine, SA<sub>14</sub>-14-2, prepared in primary hamster kidney cells, was effective in promoting an immune response in recipients without producing clinical reactions<sup>2,3,14</sup>. In several clinical trials in children, seroconversion rates were >90% after administering one dose of the SA<sub>14</sub>-14-2 vaccine. This clone was immunogenically superior to an earlier clone, SA<sub>14</sub>-5-3, in comparative vaccine trials<sup>3</sup>.

Although no apparent adverse reactions were observed in humans receiving vaccines produced in primary hamster kidney cells, alternative cell substrates that offer advantages to testing and production of future

JE vaccines were assessed. These included diploid as well as primary cells that are currently used for the preparation of several safe and effective live-attenuated vaccines. The adaptation of JE SA<sub>14</sub>-14-2 virus to primary canine kidney cells and the preparation of a first lot of vaccine are described in this paper.

## Materials and methods

### Viruses

Starting virus for passage of the JE SA<sub>14</sub>-14-2 vaccine virus in primary and diploid cells at the Walter Reed Army Institute of Research (USA) used a fifth primary hamster kidney (PHK) passage of this virus beyond the plaque purification of clone 14-2. A history of passage of the SA<sub>14</sub> virus resulting in the isolation of clone 14-2 is presented in *Table 1*. Early virus passages listed in *Table 1* were performed at the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, People's Republic of China). Terminal passages in primary canine kidney (PCK) cells were made at the Walter Reed Army Institute of Research. The parent SA<sub>14</sub> virus was derived from mouse brain passage<sup>11</sup> of the original mosquito isolate followed by three primary canine kidney (PCK) cell culture passages.

### Cells

All cells used for virus passage were aliquots from lots of frozen (liquid nitrogen) cells derived from highly characterized source tissue. These were supplied by The

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**Table 1** History of passage of JE isolate SA<sub>14</sub> and derivation of the clone 14-2

Passage	Result
Isolate from a pool of larvae of <i>C. pipiens</i> mosquitoes; 11 serial passages in weanling mice by the intracerebral route	Parent seed
100 serial passages in primary hamster kidney (PHK) cell cultures; 3× plaque purification in chick embryo (CE) cell cultures	Clone 12-1-7
2× plaque purification in CE cell cultures	Clone 17-4
1 mouse intraperitoneal passage with harvest of spleen; virus from spleen used for plaque purification in CE cells	Clone 2
3× plaque purification in CE cells	Clone 9
1 mouse subcutaneous passage and 1 plaque purification in CE cells; subcutaneous tissues were used as the source of virus for the plaque purification	Clone 9-7
6 hamster oral passages; spleens harvested for virus passage and for 2× plaque purification in PHK cells	Clone 5-3
5 suckling mouse subcutaneous passages followed by 2 plaque purifications in PHK cells; tissue from injection site used for subpassage and plaque purification	Clone 14-2
7 passages in PCK cells <sup>a</sup>	Master seed
1 passage in PCK cells <sup>a</sup>	Production seed
1 passage in PCK cells <sup>a</sup>	Vaccine lot

<sup>a</sup> Passages performed at the Walter Reed Army Institute of Research

Salk Institute, Government Services Division, Swiftwater, PA, USA. The primary canine kidney (PCK) cells were prepared from the kidneys of young (≈9-week-old) male Beagle dogs that, upon necropsy, had no evidence of any infectious or neoplastic disease. Preliminary testing of cells consisted of inoculation of broth and agar for microbial and mycoplasma sterility. Monolayers of PCK cells were also tested for the presence of canine adventitious agents using polyvalent anti-canine agent sera labelled with fluorescein isothiocyanate (FITC). Antibodies specific for canine distemper, infectious canine hepatitis, canine herpes and canine SV5 were contained in the polyvalent antisera.

Primary duck embryo (PDE) cells were derived from the eggs of certified duck flocks (SPAFAS Inc., Norwich, CT, USA). In addition to the testing for various viruses and viral antibodies in the flock, primary embryo cell cultures were established and tested for the presence of bacteria, mycoplasma and haemadsorbing agents. Cell culture material was also passaged in RK-13, DBS-FR<sub>h</sub>L-2, MRC-5 and Vero cells for detection of adventitious viral agents. All tests for contaminating microbial agents were negative.

DBS-FR<sub>h</sub>L-2 (FR<sub>h</sub>L) rhesus monkey lung diploid cells were sheeted from frozen ampoules at passage 16 and used for virus inoculation. Preliminary tests of these cells followed previously described protocols<sup>4</sup>.

### Plaque assays

JE virus titres were measured by plaque assays performed on LLC-MK<sub>2</sub> cell monolayers as previously described<sup>5</sup>.

### Tests on the master seed, production seed and vaccine

Tests to determine if adventitious agents were present in the master and production seeds and the first lot of vaccine were performed by inoculation of unclarified harvest fluids from each of these preparations into bacterial and mycoplasma media, embryonated eggs and into a variety of cell cultures and laboratory animals. Bacterial culture medium consisted of thioglycollate, soybean casein digest and Lowenstein-Jensen medium. Mycoplasma were tested for by inoculation of mycoplasma broth and agar as well as DNA staining of inoculated indicator cells. Cell cultures inoculated

included BHK-21, *Aedes albopictus* clone C6/36, 3T3, MDCK, MRC-5 and human amnion. Embryonated chicken eggs were inoculated by the allantoic and yolk sac routes. Suckling and adult mice, guinea-pigs and rabbits were also inoculated by various routes. Clarified harvest fluids were also tested for reverse transcriptase activity. Successful completion of these tests indicated that no detectable adventitious agents were present in any of the preparations.

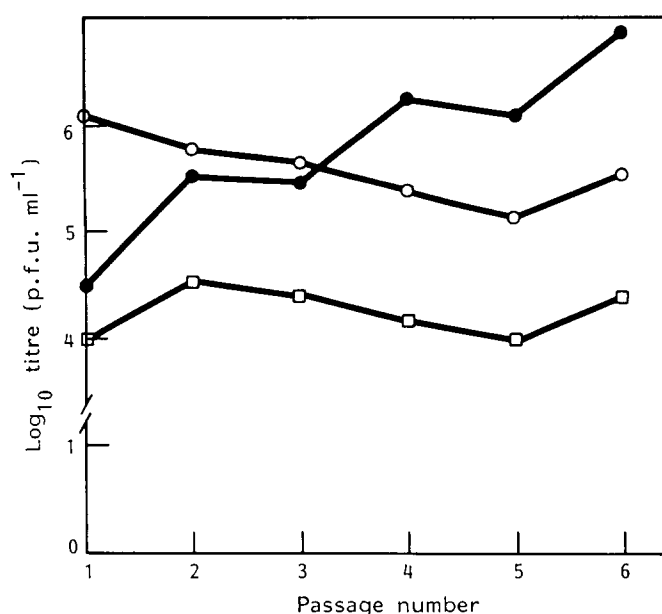
### Mouse inoculation

For virulence determinations, suckling mice were inoculated with 0.02 ml, and adult mice with 0.03 ml, virus, both by the intracerebral route. For potency assays, adult mice were vaccinated with 0.1 ml dilutions of vaccine by the subcutaneous route. Fourteen days postvaccination, mice were challenged with 100–1000 median lethal doses (LD<sub>50</sub>) of mouse-adapted JE SA<sub>14</sub> virus in a 0.3 ml dose given intraperitoneally. As part of the challenge, 0.03 ml of virus-free diluent (Eagle's minimal essential medium) was inoculated intracerebrally. Mice were observed for death daily for 21 days. A median effective dose was calculated based on the endpoint dilution of vaccine required to protect mice against the virulent JE challenge. ICR strain mice were used for all virulence and potency tests.

### Monkey inoculation and neurovirulence tests

A group of young adult rhesus monkeys were inoculated by the subcutaneous route to test for peripheral pathogenicity and immunogenicity of the vaccine and parent JE viruses. After immunization, monkeys were bled daily for virus isolation. Sera were inoculated into C6/36 mosquito cells and 14-day supernatant culture fluids were assayed for plaques. Plaque reduction neutralization tests were performed on sera obtained at 7, 14, 21 and 28 days postinoculation. The general health of all monkeys was assessed daily through the 4-week holding period.

Another group of monkeys was inoculated intraspinally and intrathalamically to determine neurovirulence of the parent and vaccine viruses. Following sedation with 5 mg kg<sup>-1</sup> Ketamine hydrochloride and 0.06 mg kg<sup>-1</sup> Rompun, both given intramuscularly, intraspinal inoculation of 0.2 ml virus or virus-free control fluids was delivered to the grey matter of the cord in the area of the lumbar enlargement. For the intracerebral in-



**Figure 1** Passage and adaptation of JE vaccine strain SA<sub>14-14-2</sub> in primary canine kidney (●), primary duck embryo (○) and DBS-FRHL-2 (□) cell cultures

jections, 0.5 ml virus or control fluids was delivered to the thalamus by inoculation into both the right and left hemispheres. Monkeys were observed daily for 17–21 days for any signs of disease or trauma. Upon completion of the holding period, monkeys were placed under deep anaesthesia and perfused with 10% buffered Formalin while undergoing exsanguination. Appropriate brain and spinal cord tissues from the right cerebral cortex, right temporal, frontal, parietal and occipital lobes, from the anterior and posterior thalamus, mid-brain, pons, cerebellum, medulla oblongata and from the cervical, thoracic and lumbar spinal cord were prepared for histological studies. Lesions found in thin, stained sections of the tissues were scored for neurovirulence using a grading scheme developed by Nathanson *et al.*<sup>6,7</sup>.

### Oligonucleotide fingerprints

The JE vaccine strain at PCK passage levels 6, 7 and 16, and the parent virus at PCK passage 3, were passaged once in C6/36 cells and viral RNA extracted from gradient-purified virions with sodium dodecyl sulphate and phenol/chloroform. RNAase T1-resistant oligonucleotides were end-labelled and separated by electrophoresis as previously described<sup>8</sup>.

## Results

### Passage of JE SA<sub>14-14-2</sub> vaccine virus in PCK, PDE and FRhL cell cultures

JE SA<sub>14-14-2</sub> (vaccine) virus at hamster kidney cell culture passage 5 was used to initiate serial passages in PCK, PDE and FRhL cell cultures. Initial growth curves of the vaccine virus in these cells indicated that the optimal time of virus harvest was 3 days after inoculation of PCK and FRhL cells and 5 days for the PDE cells. Subsequent harvests of serial passages were taken at these intervals. As shown in *Figure 1*, JE vaccine virus titres reached  $\approx 6.0 \log_{10}$  p.f.u. ml<sup>-1</sup> by passage 4 in PCK cells and maintained close to this level

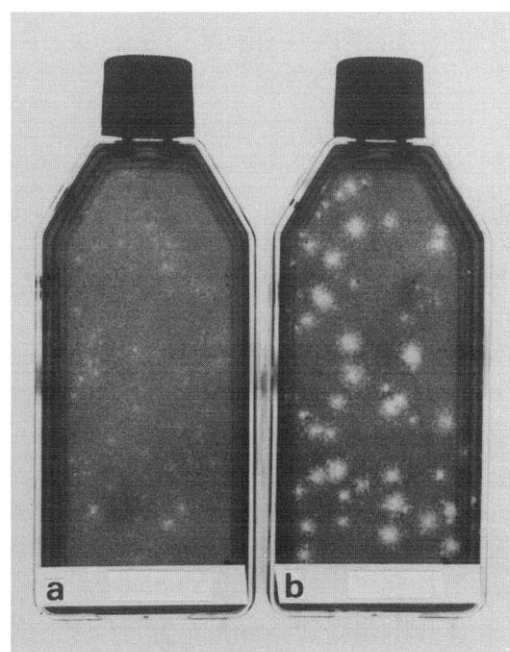
in further passages. By comparison, virus titres in the PDE and FRhL cells were lower over the time course of adaptation to these cell substrates. Superior replication and virus yields resulted in the selection of PCK cells as the cell substrate of choice for the preparation of a candidate JE master seed virus for vaccine production. The master seed was prepared at PCK passage 7 and stored in the freeze-dried state.

### Plaque morphology and temperature sensitivity of the JE vaccine and parent viruses

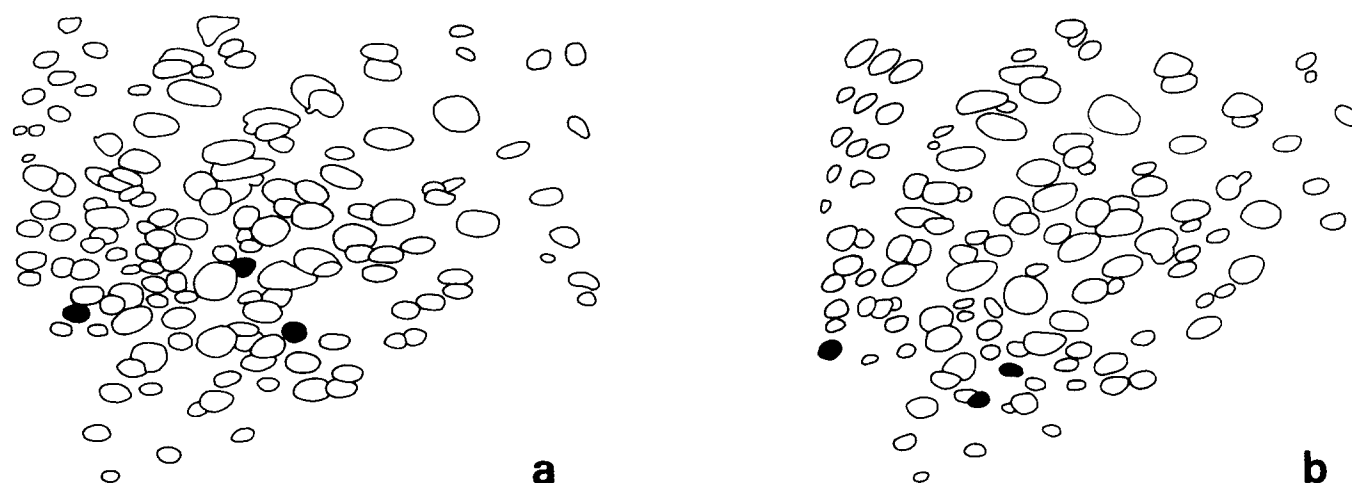
The cloned JE vaccine virus appeared to have a homogeneous small plaque morphology when plaqued in LLC-MK<sub>2</sub> cells (*Figure 2*). Also shown in *Figure 2* for comparison is the JE parent non-attenuated, uncloned virus. Typical wild-type, flavivirus plaque size heterogeneity is exhibited by the parent virus. The JE vaccine master seed (PCK-7) preparation which was used for photography, contained no large plaque virus. This was also the case for other passages of the vaccine clone grown in PCK as well as PDE and FRhL cells. An *in vitro* marker often associated with small plaque size is temperature sensitivity. Among dengue viruses, both of these markers are often associated with reduced animal virulence<sup>9,10</sup>. The data listed in *Table 2* for both the JE vaccine and parent viruses indicate that neither virus is temperature-sensitive based on plaque development in LLC-MK<sub>2</sub> cells at superoptimal temperatures ranging to 39.3°C. A reduction in plaque titre of  $>2.0 \log_{10}$  at a temperature  $>35$ – $37^\circ\text{C}$  is used as a criterion to characterize a virus as temperature-sensitive<sup>11</sup>.

**Table 2** Temperature sensitivity of the JE SA<sub>14-14-2</sub> vaccine master seed and the parent JE SA<sub>14</sub> non-attenuated virus

Virus	Log <sub>10</sub> reduction in infectivity at			
	35°C	37°C	38.5°C	39.3°C
JE vaccine	–	0.15	0.19	0.68
JE parent	–	0.14	0.14	0.76



**Figure 2** Plaque morphology of (a) JE SA<sub>14-14-2</sub> vaccine strain and (b) parent SA<sub>14</sub> viruses in LLC-MK<sub>2</sub> cell culture



**Figure 3** Nucleotide fingerprint analysis of (a) JE SA<sub>14</sub>-14-2 vaccine strain (PCK-6) and (b) parent SA<sub>14</sub> (PCK-3) viruses

**Table 3** Inoculation of mice of various ages with the vaccine clone 14-2 and the parent SA<sub>14</sub> virus to determine intracerebral neurovirulence

Virus	PCK passage	Mouse		Inoculum	Log <sub>10</sub> LD <sub>50</sub> ml <sup>-1</sup>	LD <sub>50</sub> /p.f.u.
		Age	Weight (g)			
Parent	3	1-3 days		2.0 × 10 <sup>7</sup>	7.3	1.0
		3-4 weeks	13.2		6.5	0.17
Clone 14-2	4	3-4 weeks		1.8 × 10 <sup>6</sup>	<1.7 <sup>a</sup>	<0.000 03
	7 (master seed)	1-3 days		1.9 × 10 <sup>6</sup>	6.1	0.66
		2-3 weeks	11.2		<1.5 <sup>a</sup>	<0.000 02
		3-4 weeks	17.7		<1.5 <sup>b</sup>	<0.000 02
	8 (production seed)	1-3 days		1.3 × 10 <sup>6</sup>	6.0	0.77
		3-4 weeks	13.2		<1.5 <sup>c</sup>	<0.000 02
	9 (vaccine)	1-3 days		2.4 × 10 <sup>6</sup>	6.2	0.66
		3-4 weeks	13.2		<1.5 <sup>d</sup>	<0.000 01
	15	1-3 days		5.5 × 10 <sup>5</sup>	4.9	0.23
		2-3 weeks	11.2		<1.5 <sup>b</sup>	<0.000 06

<sup>a</sup> 0/10, <sup>b</sup> 0/8, <sup>c</sup> 0/10: mice dead after inoculation with undiluted virus

<sup>d</sup> 1/10 mice died after inoculation with vaccine clone PCK-9 virus; the single mouse died 9 days postinoculation; virus recovered was vaccine virus

### Oligonucleotide fingerprints

RNA oligonucleotide fingerprints of the JE vaccine and parent viruses are presented in *Figure 3*. Most of the oligonucleotides were common for both viruses, but three oligonucleotides in the two viruses were unique for each. Higher passages of the vaccine virus, i.e. PCK-7 and PCK-16 revealed no additional nucleotide changes (data not shown). There were, therefore, six distinct oligonucleotide changes between the vaccine and parent viruses and these changes were stable on passage of this virus in cell culture. It was not possible to conclude that the nucleotide changes which generated these differences were the only ones which occurred during passage of the vaccine virus. Additionally, these changes could not be linked to any functional or biochemical marker for this attenuated virus. However, the fingerprints could be used to differentiate the attenuated and non-attenuated JE viruses and to identify genome changes that may occur in the vaccine virus during use of this vaccine in man.

### Lack of virulence of the JE vaccine virus in young mice

The JE SA<sub>14</sub>-14-2 vaccine virus was attenuated for young mice, causing no symptoms or death in mice >2 weeks of age receiving the virus intracerebrally<sup>2</sup>. Various passage levels of the JE vaccine virus were inoculated intracerebrally (i.c.) into weanling and young

**Table 4** Monkey viraemia and immune response after subcutaneous inoculation with the parent JE SA<sub>14</sub> virus and the vaccine clone 14-2 production seed

Monkey No.	Virus inoculated	Viraemia	Neutralizing antibody at days postinoculation	
			0	28
483	Parent	0	<10 <sup>b</sup>	400
626	Production seed	0	<10	80
628	Production seed	0	<10	160
717	Production seed	0	<10	160

Parent seed was inoculated at 7.5 × 10<sup>5</sup> p.f.u. and production seed at 3.7 × 10<sup>5</sup> p.f.u. To estimate viraemia, monkeys were bled on 12 consecutive days and serum inoculated into C6/36 mosquito cells for amplification. After 14 days, the supernatant fluids were assayed for virus. Neutralizing antibody is expressed as the reciprocal of the serum dilution resulting in 50% plaque reduction

adult mice and observed for signs of encephalitis over a 2-week period. As shown in *Table 3*, none of the PCK-passaged JE viruses caused encephalitis or death in these mice. The JE non-attenuated parent virus routinely caused death in similar groups of mice in 7 days. The LD<sub>50</sub>/p.f.u. ratio for the parent virus was 0.17 in 3 to 4-week-old mice while this ratio for the vaccine virus was <0.000 02 in similar mice. In newborn mice this ratio was increased for the vaccine virus to 0.66. The JE vaccine virus, after passage in PCK cells for a total of 15 passages, was still clearly attenuated for mice inoculated i.c. These results are similar to those pub-

**Table 5** Neurovirulence study in rhesus monkeys inoculated intracerebrally and intraspinally with parent SA<sub>14</sub>, clone 14-2 production seed and virus-free control fluids

Inoculum	No. of monkeys	No. with CNS disease	Total No. of CNS sites observed	No. of lesions with grade		
				V-1	V-2	V-3
Control fluids	2	0	28	0	0	0
Parent virus	2	2	28	4	19	3
Production seed	10	0	140	27	0	0

**Table 6** Potency assay of the JE vaccine clone 14-2 virus in adult mice

Inoculum	ED <sub>50</sub>	Challenge dose (LD <sub>50</sub> )
PCK-4	1900*	>1000
PCK-7 (master seed)	3200	500
PCK-9 (vaccine)	2000	>1000

ED<sub>50</sub>, dilution of virus that protected 50% of a group of mice against challenge with the neurovirulent parent SA<sub>14</sub> virus

lished for the same JE clone passaged in hamster kidney cells<sup>2</sup>.

### Monkey peripheral and intracerebral virulence

Monkeys inoculated subcutaneously with either the parent or vaccine viruses did not show signs of CNS disease and remained healthy over a 28-day observation period. Although virus could not be recovered from the blood of these monkeys for 12 days following inoculation, production of neutralizing antibodies after 4 weeks indicated that virus infection had taken place (Table 4). Monkeys receiving combined intraspinal/intrathalamic inoculations were observed for a period of 17–21 days. The two monkeys that received the parent virus both showed signs of CNS disease 6 days following inoculation. Partial paralysis in the lower limbs was observed on day 6, followed by complete immobility and total paralysis of all limbs on day 7. Both were killed on day 7. Neurovirulence lesions in these monkeys occurred in all brain tissue examined except for the cerebellum. Lesions were graded minimal (V-1), mild (V-2) or moderate (V-3). The numbers of lesions for each of the assigned grades are listed in Table 5. Degeneration/necrosis and loss of neurons with an accompanying minimal to mild leucocytic/microglial cell accumulation adjacent to many small blood vessels and neurons in the brain and spinal cord characterized the histopathological changes that occurred in monkeys that received the JE parent virus. The 10 monkeys receiving the JE vaccine virus (production seed) were free of neurovirulence lesions in most areas of the brain and spinal cord. The grade assigned to the lesions never exceeded V-1 where neuronal degeneration or necrosis was absent and a minimal number (1–2 in a structure or region) of lymphohistiocytic cuffs were found in blood vessels.

### Mouse potency

The efficacy of vaccination with JE SA<sub>14</sub>-14-2 can be determined by a mouse assay using one dose of vaccine followed by a peripheral challenge. Table 6 shows assays for three passage levels of the JE SA<sub>14</sub>-14-2 vaccine virus. The vaccine potency endpoint is expressed as the median effective dose, ED<sub>50</sub>, i.e. a dilution of vaccine that is required to protect 50% of mice challenged with virulent JE virus. This dilution ranged from 1900 to

3200 for the three preparations. Translated into infectious units, these dilutions contained 56 to 120 p.f.u. JE virus. Therefore, in addition to plaque assays for determining vaccine potency, a small animal model is available for measuring vaccine efficacy. The validity of the mouse model is substantiated by the susceptibility of the mouse to infection with JE and the development of encephalitis after a peripheral challenge.

### Discussion

The JE SA<sub>14</sub>-14-2 primary hamster kidney cell vaccine has already undergone evaluation in human subjects and the results indicate that this vaccine candidate is sufficiently safe and efficacious to continue further laboratory, animal and human studies. The present studies indicate that the SA<sub>14</sub>-14-2 vaccine virus can replicate to high titre in PCK cells without apparent phenotypic change. The PCK cells were prepared from young, pedigree dogs that were raised in closed colonies specifically designated for scientific studies. Each dog that was killed for kidney tissue was examined for signs of disease during necropsy. In addition, each lot of cells that was derived from a pair of kidneys was tested for the presence of adventitious agents after the cells were frozen in aliquots in liquid nitrogen. These controls on the virus substrate are essential for producing vaccines that are contaminant-free. The use of bank-frozen, pre-tested cells such as these ensure that a product made from the cells is not only safe for human use but also provides a measure of quality control for the product.

A vaccine containing a modified encephalitogenic agent such as JE must be proven safe prior to human immunization. Although the hamster-kidney-cell-passaged JE vaccine appears to be attenuated for humans, introduction to the PCK substrate may modify the vaccine virus. An *in vitro* measure of phenotypic change is the emergence of plaque variants, morphologically different from those found in the original cloned virus seed. Comparison of plaques from various passages of the PCK-grown JE SA<sub>14</sub>-14-2 virus, up to and including the vaccine preparation, with the original hamster kidney-grown virus, indicated that no phenotypically changed virus populations were evident in the PCK-passaged material.

Another check on viral genome change is nucleotide fingerprint analysis of the vaccine clone. This kind of analysis has been used to track nucleotide changes for other viruses during host transmission<sup>8,12</sup>. In the case of the JE vaccine virus, it was especially important to detect any changes resulting from cell culture passage that may be associated with loss of attenuation. The stability of the JE vaccine virus was evident from the lack of fingerprint changes over a wide range of cell culture passages. A large number of nucleotide changes found in comparisons of variant and parent viruses is

not uncommon. Yellow fever 17D vaccine strain also contains a number of unique nucleotides when compared to the parent Asibi strain<sup>13</sup>. Sequencing of the JE vaccine virus genome and parent genome will allow future assignment of base changes to structural or non-structural viral proteins and eventually correlation with modifications of replicative function that result in an attenuated phenotype. Lack of temperature-dependent restriction of viral replication does not seem to be important for the observed animal attenuation of this virus. Lack of the ts phenotype, also the case for yellow fever 17D vaccine, suggests that some form of host restriction has occurred for these viruses. Both of these viruses have similar histories of extensive passage in hosts and host tissue that are not part of the natural infection cycle.

The mouse is very useful as an experimental animal for JE viral encephalitis<sup>2</sup>. The JE SA<sub>14</sub>-14-2 vaccine virus is not encephalitogenic in young mice inoculated i.c. while the parent SA<sub>14</sub> virus causes a lethal encephalitis. Higher passage in PCK cells does not select for virulence, as the vaccine virus after PCK passage 15 did not cause sickness or death in another group of i.c.-inoculated mice.

Peripheral virulence with JE viruses is hard to demonstrate in either small animals or in primates. As was shown in this study, large doses of intracerebral-virulent SA<sub>14</sub> virus did not cause encephalitis in monkeys when given subcutaneously. Even viraemia could not be demonstrated in these animals. The mouse potency assay uses an augmented peripheral challenge by traumatizing the brain with sterile diluent. The sham intracerebral inoculation probably allows passage of virus and infection in the brain which results in encephalitis and death in these animals.

Neurovirulence tests in monkeys also indicated that the vaccine virus had minimal encephalitogenic potential. CNS disease along with histopathologic changes in brain and spinal tissues of JE parent virus-inoculated monkeys were typical of other non-attenuated viruses of the JE virus complex of the flaviviruses<sup>6</sup>. Neurovirulence testing of production seeds or vaccine lots of YF vaccine is required by regulatory authorities to prove safety. This standard should apply to all live-attenuated JE vaccines intended for human use.

The stability and lack of animal virulence for the JE SA<sub>14</sub>-14-2 vaccine virus after adaptation and growth in PCK cells makes this virus a prime candidate for JE vaccination. Small scale human testing for safety and immunogenicity may now proceed for the new JE PCK vaccine.

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