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Title	Japanese Encephalitis Purified Vaccine
Author(s)	Takaku, Keisuke; Yamashita, Takayoshi; Osanai, Takeichi et al.
Citation	Biken journal : journal of Research Institute for Microbial Diseases. 1968, 11(1), p. 25-39
Version Type	VoR
URL	https://doi.org/10.18910/82878
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BIKEN JOURNAL Vol. 11, 25-39, 1968

JAPANESE ENCEPHALITIS PURIFIED VACCINE[1]

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(Received February 8, 1968)

SUMMARY A procedure for purification of Japanese encephalitis (JE) vaccine from infected mouse brains is described. The method is based on two cycles of ultracentrifugation of active carbon-treated protamine extract of infected brain homogenate. This gives a very effective vaccine containing very little protein nitrogen. Purified vaccine is rather labile in phosphate-buffered saline, but can be stabilized by adding gelatine to TC Medium 199 buffered with phosphate. The absence of encephalitogenic substances in the vaccine was demonstrated.

Preliminary successful results on similarly purified vaccine from brains of mice infected with the current JaGAr#01 strain of Japanese encephalitis virus, instead of the usual Nakayama vaccine strain, are also described.

INTRODUCTION

Since 1954 vaccination of humans against Japanese encephalitis (JE) has been put into practice in Japan. The first vaccine employed was the formalin-inactivated supernatant of infected mouse brains. Since then extensive efforts have been made to prepare more potent vaccine and to eliminate possible contaminating substances causing allergic encephalomyelitis from the vaccine. The standard level of vaccine potency stipulated by the Ministry of Health and Welfare of Japan has been raised almost every year. In 1959 the Minimum Requirements of this vaccine were revised to restrict the total nitrogen content to less than 0.4 mg/ml. Later protamine sulfate and active carbon- or kaolin-treated vaccine was developed. In 1962 the total nitrogen content of the vaccine was limited to less than 0.2 mg/ml.

In 1965 two kinds of further purified vaccine prepared from infected mouse brains were introduced. One was devised by investigators at the Nippon Institute for Biological Science and was prepared by protamine treatment of

[1] Presented in part at the 13th General Assembly of the Society of Japanese Virologists, Nagasaki, 1965.

ethanol-precipitated viruses. The other was developed in this Institute and was prepared by ultracentrifugation of the protamine- and active carbon-treated viruses. After extensive examinations and field surveys by the respective institutes followed by trails by the Committee on the Japanese Encephalitis Vaccine, which has been organized by experts throughout Japan, these vaccines have both been recognized as being better than the previous protamine vaccine (KAWAKITA, 1966). Based on the results obtained by this Committee, the Minimum Requirements of JE vaccine were again revised to restrict the hot trichloracetic acid (TCA)-precipitable nitrogen to less than 0.02 mg/ml.

Many arboviruses have been recognized throughout the world to cause diseases similar to JE, and most of them are known to multiply in mouse brains in sufficiently high titer to prepare vaccine with high potency. However, fear of possible contamination of the vaccines with encephalitogenic substances has prevented the practical use of mouse brain vaccines even when these have been recognized to be effective. The method of purification of JE vaccine described here gives promise for control of a good number of arbovirus diseases throughout the world.

MATERIALS AND METHODS

1. *Virus*

Japanese encephalitis virus, Nakayama (exactly, Nakayama-Yoken) strain was obtained from the National Institute of Health of Japan and was maintained in this Institute by alternating mouse passage with storage in liquid nitrogen. The current strain of Japanese encephalitis virus, JaGAr#01 strain, isolated from mosquitoes in 1959 (MATSUYAMA *et al.*, 1960), was also obtained from the National Institute of Health of Japan, and was maintained like the Nakayama strain.

2. *Mice*

For vaccine production, four week old albino mice of mixed-breed and both sexes were purchased from local dealers. For titration of virus (mouse LD_{50}) and of vaccine potency (mouse ED_{50}) or the neutralization test by plaque reduction, inbred albino mice, strain dd-Y were used.

3. *Centrifuges*

For separating the supernatant from the brain homogenate and after the protamine- or active carbon-treatment, bucket type refrigerated centrifuges, type H-250, Kokusan Enshinki Co., Ltd. were used.

To spin down the virus particles, preparative ultracentrifuges, Model 40P, 55PA or 55P-2 with RP-21 rotors, from Hitachi Ltd. were used.

4. *Homogenizers*

For homogenizing mouse brain, large scale homogenizers, NK mixers from Nihon Seiki Seisakusho Co. were used. Virtis 45 homogenizers from the Virtis Co., Inc. were used for resuspending virus pellets after ultracentrifugation.

5. *Chemicals*

Protamine sulfate was obtained from Nutritional Biochemicals Corp. Active carbon was obtained from Wako Pure Chemical Industries, Ltd. TC Medium 199 W/O bicarbonate and phenol red, dried, and Bacto-gelatin were obtained from DIFCO Laboratories.

The medium for suspension of the final vaccine (0.02 per cent gelatin in medium 199 with M/150 phosphate buffer, pH 7.2 (Gel-PB-199)) was sterilized by filtration through a Millipore filter, type HA, from Millipore Filter Corp.

For tissue culture, trypsin, Bactoagar, Bacto-yeast extract, Bactotryptose phosphate broth, dehydrated, and TC Medium 199, dried, were obtained from DIFCO Laboratories. Lactalbumin hydrolysate was obtained from Nutritional Biochemicals Corp. Calf sera were obtained from a local slaughter house, and those containing no inhibitor to JE virus were selected for use.

6. *Antisera*

a) Anti-mouse brain serum: Rabbits were immunized 3 times intramuscularly at intervals of 4 weeks with a fresh homogenate of 0.5 g (wet wt) of normal mouse brain in complete Freund's adjuvant. The rabbits were bled 3 weeks after the last injection.

b) Anti-ultracentrifuge-purified vaccine serum; Rabbits were immunized intravenously 3 times, every other day with 1 ml of 7 times concentrated purified vaccine. Then after an interval of 2 weeks,

immunization was repeated 3 more times. Two weeks after the last injection, rabbits were bled. Antisera were inactivated by heating at 56°C for 30 minutes and then stored at −20°C without any preservative.

7. *Encephalitogenic basic protein form mouse brain tissue*

Basic protein, which was recognized as a causative antigen of allergic encephalomyelitis (PETERSON, 1966) was extracted and partially purified according to the method of KIES (1965).

8. *Ouchterlony technique*

Supporting gel was made from a mixture of 0.35 per cent each of Special Agar Noble, DIFCO and agar from " ogonori " (*Gracilaria verrucosa*), which was obtained through the generosity of Dr. S. Tanabe of the Department of Bacteriology, Osaka University Medical School, in phosphate buffered saline, pH 7.2.

9. *Titration of JE virus*

a) Plaque forming units (PFU) were titrated on a chick embryo fibrobalst (CEF) culture hy the method of PORTERFIELD (1960) with slight modifications (IGARASHI *et al.*, 1963).

b) Mouse LD_{50}: The virus sample was serially diluted ten-fold, usually from 10^{-5} to 10^{-9}, with phosphate buffered saline, pH 7.4. A group of 10 mice were inoculated intracerebrally with 0.03 ml of each dilution. The mice were observed for 14 days. At the end of the test the LD_{50} was calculated by the Reed-Muench accumulation formula.

10. *Potency titration of vaccine*

a) Mouse ED_{50} was estimated by the method described in Minimum Requirements of Biological Products, Japan, 1967. The test sample was diluted to obtain 4 serial 4-fold dilutions. Each dilution was inoculated intraperitoneally into more than 10 mice in a dose of 0.5 ml. The inoculation was repeated 4 times at 2 day intervals. The mice were challenged intracerebrally on the 14th day from the first immunization with JE virus, Nakayama strain, unless otherwise stated, in a dose of 0.03 ml, containing an appropriate LD_{50} of the virus. The challenged mice were observed for 21 days. Fifty per cent survival of the vaccinated mice was calculated by the Reed-Muench accumulation formula. As controls, animals were treated with the standard vaccine from the National Institute of Health of Japan or our own reference vaccine.

b) Neutralization test by plaque reduction was carried out by the method developed by the Committee on the Japanese Encephalitis Vaccine (OYA, 1966; OYA *et al.*, 1966; KUNITA *et al.*, 1966; NOBUTO *et al.*, 1966). The test sample was diluted with phosphate buffered saline, pH 7.4 to obtain 3 serial 4-fold dilutions. Each dilution was inoculated twice intraperitoneally into more than 10 mice in a dose of 0.5 ml with an interval of 7 days between the injections. On the 15th day after the first immunization mice were bled by cardiac puncture. The individual blood samples were immediately mixed with an equal volume of phosphate buffered saline, pH 7.4. The diluted blood samples were stored at 4°C overnight to allow complete coagulation. The diluted serum was separated by centrifugation and diluted further with an equal volume of LH medium containing 5 per cent calf serum, and was inactivated by heating at 56°C for 30 minutes. This diluted serum was defined as 1/10 diluted serum, and was stored at -20°C until use. Volumes of 0.4 ml of the " 1/10 diluted " serum were mixed with an equal volume of an appropriate dilution of JE virus to give approximately 100 plaques per dish. Each mixture was incubated at 37°C for 1.5 hours. After incubation the mixture was inoculated onto a one-day monolayer of CEF culture in a Petri dish of 70 mm diameter. The plate was incubated at 37°C for 1.5 hours, and then was overlayed with 8 ml of 1 per cent agar containing Earle's medium, 0.5% lactalbumin hydrolysate and 0.1% yeast extract. Then the plate was incubated further under a 5 per cent CO_2 atmosphere at 37°C for 48 hours. After this period it was again overlayed with 4 ml of 1 per cent agar containing neutral red in Earle's medium, and was incubated at 37°C in a CO_2 incubator. At the 24th and 48th hour, the plaques were counted. As control series, virus suspensions without serum, and with known positive and negative sera were included. The plates showing less than 50 per cent of the number of plaques in the plate of control virus were recorded as showing a positive reduction. The neutralization ED_{50} ($NTED_{5c}$) was calculated by the Reed-Muench accumulation formula.

11. *Hemagglutination inhibition test*

This was carried out by the method of CLARK and CASALS (1958) but the serum specimen was treated with kaolin instead of acetone to remove inhibitor

(OYA, 1964).

12. *Nitrogen determination*

The content of TCA-precipitable nitrogen in the samples was determined as follows: The sample was mixed with trichloracetic acid to a concentration of 5 per cent, and then heated in a boiling water bath for 15 minutes. The insoluble material was collected by centrifugation and its nitrogen content was measured by the micro-Kjeldahl method (Minimum Requirements of Biological Products, Japan, 1967).

13. *Electron microscopy*

Virus preparations were examined by the negative staining method using phosphotungstate (BRENNER and HORNE, 1959) in a Hitachi electron microscope, model HU-11B.

RESULTS

1. *Method of preparation of the vaccine*

As shown in Fig. 1, two alternative methods can be used for virus purification, i.e., purification of inactivated viruses and inactivation of purified live viruses. These methods give comparable purities and potencies.

Mice were inoculated intracerebrally with JE virus, Nakayama strain. After full development of the disease, mice were killed, and their brains were taken out and homogenized in phosphate buffered saline, pH 8.0, at a concentration of 20 per cent brain tissue. After low speed centrifugation, the supernatant was treated with protamine sulfate at a concentration of 1.8 mg/ml for 90 minutes in an ice water bath. Then, the protamine precipitate was removed by centrifugation.

In the " live virus procedure " the protamine extract was further treated with active carbon at a concentration of 1 per cent (w/v) in an ice water bath for 15 minutes to adsorb some non-viral substances. The carbon was removed by centrifugation. For higher purification the supernatant should be filtered through a Millipore filter membrane, type HA. The clarified virus suspension was centrifuged at $59{,}000 \times g$ for 6 hours in a prepatative ultracentrifuge. Then the supernatant was slowly removed until 1/20 volume of the bottom layer of supernatant was left. The pellet and the bottom 1/20 portion of super- natant were collected and homogenized in 0.02 per cent gelatin in phosphate buffered saline, pH 7.2, (Gel-PBS) in a Virtis 45 homogenizer. The resulting virus suspension was again ultracentrifuged in the same condition. The pellet and the bottom layer of supernatant were homogenized. The virus suspension was then mixed with 0.5 M glycine, and formalin was added to a concentration of 0.05 per cent. Glycine was necessary for maintaining a high immunogenic potency of the purified virus during the inactivation period. The formalin inactivation was carried out at about 4°C for 60 days. During this period the inactivation was checked at intervals by intracerebral injection of the suspension into suckling mice. Usually inactivation was complete within 40 days.

After filtration through a Millipore filter membrane, type HA, the virus suspension was diluted to the desired concentration with TC Medium 199 containing 0.02 per cent gelatin and M/150 phosphate buffer, pH 7.2 (Gel-PB-199).

In the " killed virus procedure " the viruses were inactivated by formalin following to the protamine-treatment. In this case the addition of glycine was unnecessary. Before active carbon treatment the inactivated virus suspension was passed through a sintered glass filter, grade M, to remove the flocculent precipitate formed during the inactivation period. The virus particles were purified by two cycles of ultracentrifugation under the same condition used in the live virus procedure. For routine production the killed virus procedure was used, as shown in the left half of Fig. 1. The final vaccine preparations contained 2–5 μg of hot TCA-precipitable nitrogen per ml as compared to the amount of 20 μg or less per ml stated for the Minimum Requirements of Biologic Products by the Ministry of Health and Welfare of Japan.

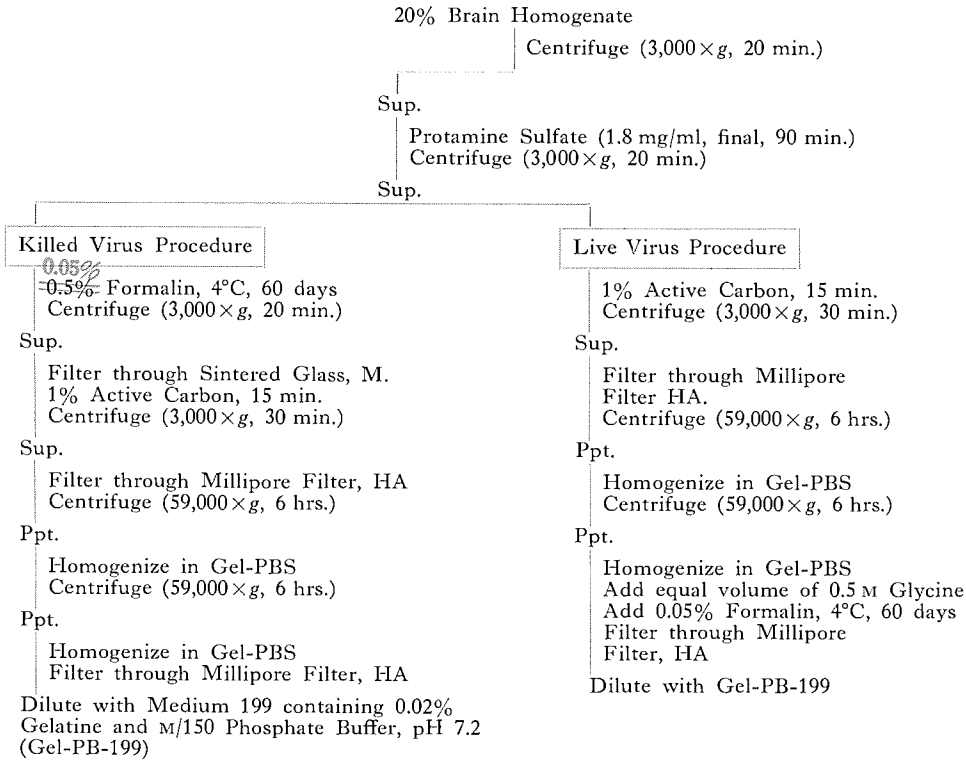


FIGURE 1 Procedures for Purification of Japanese Encephalitis Vaccine.

TABLE 1 *Virus titers and protein contents following each purification step**

Treatment		with active carbon treatment		without active carbon treatment	
		Bottom 1/20 sup.+Pellet	Pellet only	Bottonm 1/20 sup.+Pellet	Pellet only
Protamine treatment	PFU/ml	11.1×10^8	17.9×10^8	7.8×10^8	12.5×10^8
	μg protein/ml	3,397	3,599	3,393	3,223
	PFU/μg protein	33	60	28	38
Active carbon treatment	PFU/ml	11.3×10^8	16.7×10^8		
	μg protein/ml	1,283	1,427		
	PFU/μg protein	87	119		
Single ultracentrifugation	PFU/ml	51.8×10^8	77.7×10^8	102.5×10^8	77.5×10^8
	μg protein/ml	730	105	2,053	160
	PFU/μg protein	1,960	6,686	437	6,829
	Per cent PFU recovery	79	34	95	67

* Average values of several experiments.

2. *Purity of the vaccine*

Table 1 shows the results obtained by the live virus procedure using a single cycle of ultracentrifugation. Results are expressed as the average values of several experiments. As shown in this table, the active carbon step yielded material for ultracentrifugation which was about twice as pure as the original protamine extract.

When only the pellet obtained by ultracentrifugation was used for vaccine, the purification ratio was greatly increased but the loss of virus was also significant. To avoid loss of virus and increase purity, two cycles of ultracentrifugation of the pellet plus the bottom 1/20 of the supernatant obtained at each cycle was adopted as the standard method.

Fig. 2 shows an Ouchterlony plate of diffusion of samples at various purification steps against anti-normal mouse brain rabbit serum. All the antigen samples were of a stages of purification of a single protamine extract, and their concentrations were adjusted to 6.5 times that of the final vaccine product by ultrafiltration or dilution. As shown in this figure, some antigens in the mouse brain homogenate and the samples of earlier purification steps cross-reacted with mouse serum proteins. As the purification proceeded, the antigens, which reacted with anti-normal mouse brain serum, were lost. The final product in well No. 6 contained little of these antigens.

Fig. 3 shows an electron micrograph of the final vaccine seen by the negative contrast method (BRENNER and HRONE, 1959). Many typical virus particles of 40 mμ diameter and smaller entities identified as disintegration products of the virus particles can be seen (FUKAI and KITANO, 1968).

3. *Immunogenic potencies of the vaccine*

Fig. 4 shows the distribution of the potencies of our products for 1966 and 1967, expressed as the $\log_4$ of the mouse ED_{50}'s plotted against the $\log_{10}$ of the challenge virus doses in LD_{50}. The open circles represent our products, and the solid circles are those of the standard

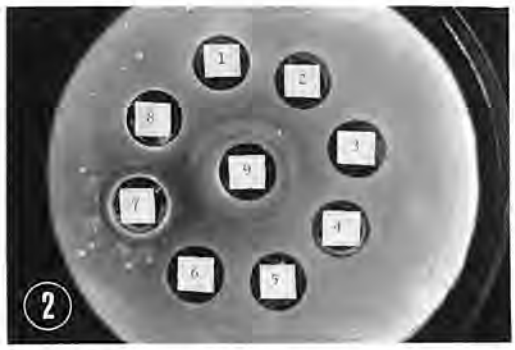


FIGURE 2 Immunodiffusion analysis of samples from various purification steps.

1: Protamine extract
2: Active carbon-treated protamine extract
3: Supernatant from the first ultracentrifugation
4: Pellet and bottom 1/20 of the supernatant from the first ultracentrifugation
5: Supernatant from the second ultracentrifugation
6: Pellet and bottom 1/20 of the supernatant from the second ultracentrifugation
7: Normal mouse serum
8: Supernatant from normal mouse brain homogenate
9: Anti-normal mouse brain rabbit serum (RMB-2)

The concentrations of antigen samples were adjusted to 6.5 times that of the final vaccine product by ultrafiltration or dilution, except in the cases of the supernatant from the 20 per cent normal mouse brain homogenate and normal mouse serum, neither of which were either concentrated or diluted.

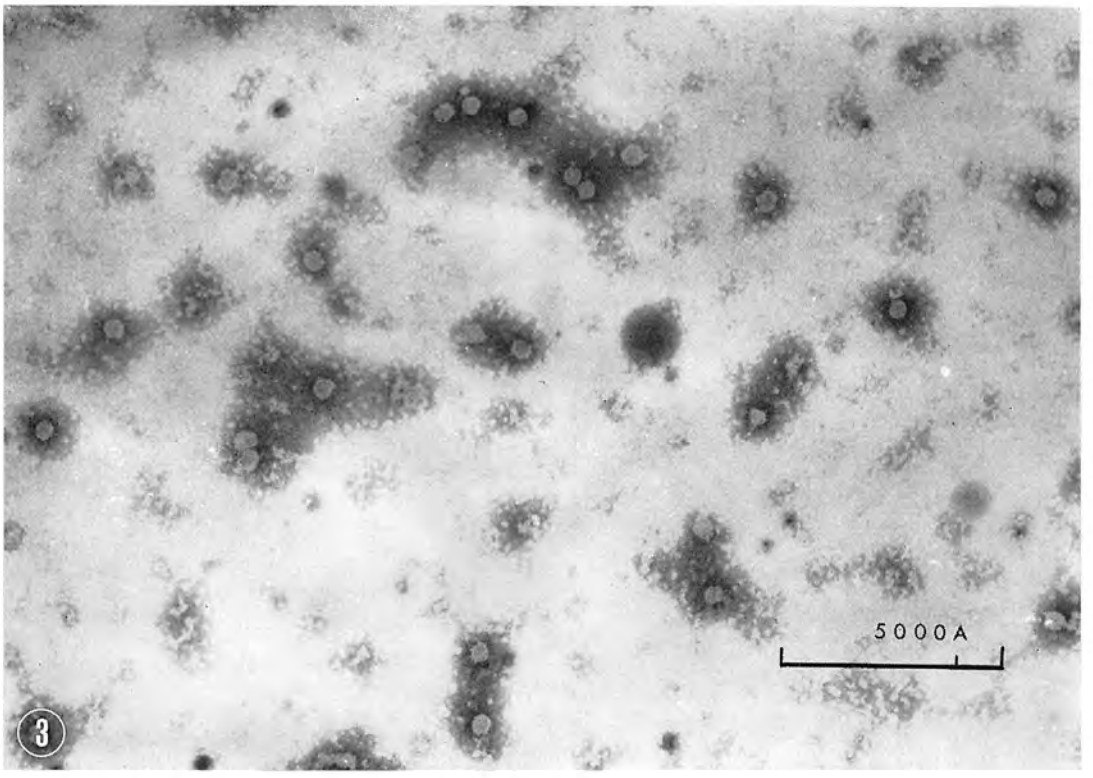
FIGURE 3 Electronmicrograph of the final product of the ultracentrifuge-purified JE vaccine. Negative stain with phosphotungstate. ⟶

FIGURE 4 Mouse ED_{50} of ultracentrifuge-purified JE vaccine prepared for 1966 and 1967.

○: Ultracentrifuge-purified vaccine,
●: Standard reference vaccine from National Institute of Health of Japan. ⟶

vaccines of each year from the National Institute of Health of Japan. The solid line represents the proposed standard distribution line for the standard vaccine of the National Institute of Health (OGATA and OYA, 1965).

As shown from the scattered distribution of the plots of the standard vaccines, the estimation of vaccine potency by mouse ED_{50} is


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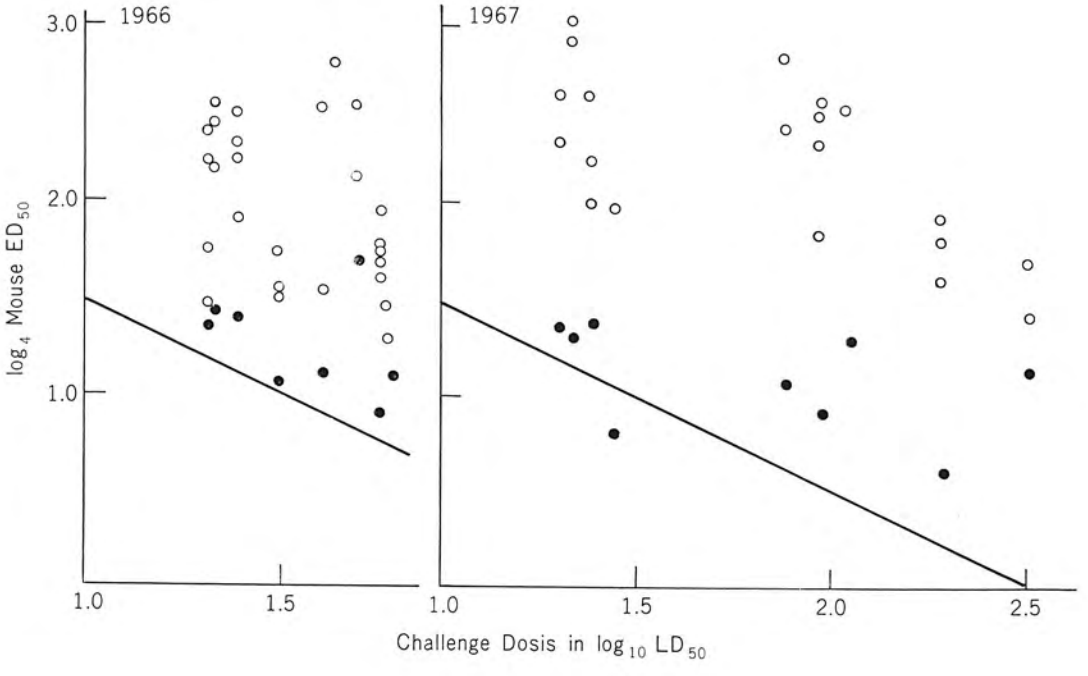

1966
1967
3.0
2.0
1.0
log4 Mouse ED50
1.0
1.5
1.0
1.5
2.0
2.5
Challenge Dosis in log10 LD50

TABLE 2 *Effect of the suspending medium on the stability of ultracentrifuge-purified vaccine stored at 2° to 5°C*

Suspending medium	Potency at the end of indicated month after preparation*				
	0	2	4	6	12
M/75 phosphate buffered saline	1.9	1.6	<1.0	<1.0	<1.0
0.02% Gelatin in M/75 phosphate buffered saline	1.9	2.1	2.6	2.1	1.6
0.02% Gelatin- and M/150 phosphate buffer in TC Medium 199	1.9	2.3	2.8	2.6	2.5
Reference protamine vaccine**	1.9	1.9	2.0	2.0	2.1
Challenge virus doses in mouse LD_{50}	26	24	16	20	14

This test lot of vaccine contained 2.1 μg hot TCA-precipitable nitrogen per ml.

* Expressed as the $\log_4$ of the mouse ED_{50}.

** Standard vaccine from the National Institute of Health of Japan distributed for 1965 production season.

not very exact. However, most of the plots for our ultracentrifuge-purified vaccines are far above the proposed line.

4. *Stability of the vaccine*

The potency of the old type protamine vaccine was maintained in phosphate buffered saline, as shown in Table 2. As the purity was improved, however, the stability was lost abruptly, especially when the protein content (hot TCA-precipitable nitrogen content) in the vaccine was less than 15 μg per ml. Many chemicals were tested for their ability to maintain the potency of purified vaccine. As shown in this table, the addition of gelatin prevented the loss of potency to some extent. Several amino acids alone and in combination had some effect. However, Gel-PB-199 was finally found to give the best results of the chemicals tested.

One of our products, manufactured during the 1966 production season, was also used as our own reference vaccine in mouse ED_{50} tests during the 1967–1968 production seasons. This lot of the vaccine contained 2.5 μg of hot TCA-precipitable nitrogen per ml. As shown in Fig. 5, the potency of this vaccine has been well maintained at 4°C over a period of more than 20 months.

FIGURE 5 Mouse ED_{50} of a lot of the product prepared in 1966.

This lot of vaccine was put in vials on February 18, 1966, and was stored at 2° to 5°C during the period. The hot TCA-precipitable nitrogen content was 2.5 μg/ml.

The letters showing plots represent the tests completed in the months shown below.

A : March, 1966	K : March, 1967
B : April, 1966	L : April, 1967
C : July, 1966	M : May, 1967
D : August, 1966	N : June, 1967
E : September, 1966	O : July, 1967
F : October, 1966	P : August, 1967
G : November, 1966	Q : September, 1967
H : December, 1966	R : October, 1967
I : January, 1967	S : November, 1967
J : February, 1967	T : December, 1967

5. *Elimination of encephalitogenic substances from the vaccine*

When vaccine is prepared from brain tissue, there is a danger that it may be contaminated with substances which could cause allergic encephalomyelitis in persons immunized with it. Since administration of JE vaccine started in 1954, no compatible case of allergic encephalomyelitis resulting from administration of JE vaccine has been reported, even before ultracentrifuge purified vaccine was introduced. This problem of possible contamination by

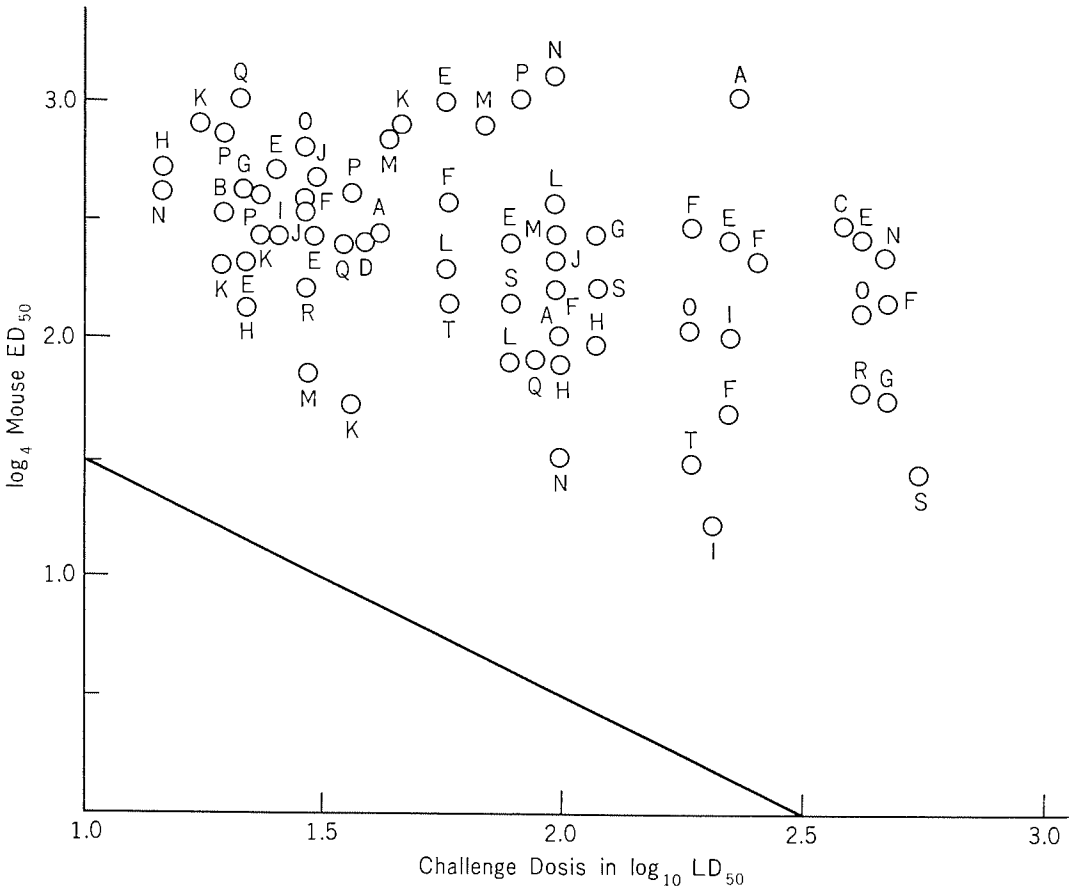


TABLE 3 *Occurrence of allergic encephalomyelitis in cynomolgus monkeys after single injection of various antigens*

Antigen	mg N/ml*	Dose, ml	Clinical paralysis	Histological lesion	
				Brain	Spinal cord
Normal rabbit spinal cord homogenate	1.0	1.0	4/4	4/4	2/4
Normal mouse brain homogenate	10.0	1.0	0/4	1/4	2/4
	0.98	1.0	0/4	0/4	0/4
50× concentrated** protamine vaccine	5.9	1.0	0/4	0/4	0/4
50× concentrated** ultracentrifuge-purified vaccine	0.12	1.0	0/12	0/12	0/12

Antigen was mixed with an equal volume of adjuvant containing 85% Drackeol, 15% Arlacel A and 2 mg/ml *Mycobacterium tuberculosis*, strain Aoyama B, and injected intramuscularly into the femoral region on either side.

* Total nitrogen (for brain and spinal cord) or hot TCA-precipitable nitrogen (for vaccines).

** Concentrated by lyophilization.

encephalitogenic substances is still very important, however, when it is considered that the number of vaccinated people has recently been increasing rapidly. Therefore, this point was re-examined using cynomolgus monkeys as test animals.

The antigens used were a spinal cord homogenate from rabbits, a brain homogenate from mice and old type protamine vaccine and new ultracentrifuge-purified vaccine. The vaccines were concentrated 50 times by lyophilization. All the antigens were injected with Freund's complete adjuvant. As shown in Table 3, all the monkeys receiving rabbit spinal cord preparation containing 1 mg total nitrogen developed clinical paralysis on or after the 14th day after injection. Histopathological examination showed that these animals had lesions in the cerebrum, thalamus, cerebellum and pons. Two of the 4 monkeys in this group also showed lesions in the spinal cord. No monkey showed clinical paralysis after injection of mouse brain homogenate containing 10 mg total nitrogen. However, when animals were examined on the 35th day, some of them showed lesions in the brain and spinal cord.

No animal, receiving either kind of concentrated vaccine, showed clinical paralysis or histopathological lesions.

Recently, a basic protein extracted from the tissues of the central nervous system of various mammals at an acidic pH has been shown to produce allergic encephalomyelitis in experimental animals (Robez-Einstein *et al.*, 1962; Kies, 1965; Peterson, 1966). This antigen prepared from normal mouse brain can develope a definite precipitation line against anti-normal mouse brain serum, but not against anti-ultracentrifuge-purified vaccine serum, as shown in Fig. 6. On the other hand, concentrated ultracentrifuge-purified vaccine occasionally developes a few faint lines against anti-normal mouse brain serum. However, neither fusion nor spur-formation is observed between the precipitation lines formed by the vaccine and that by encephalitogenic basic protein, and only the clear intersection between them can be observed. These results indicate the absence of encephalitogenic basic protein in the purified vaccine preparation.

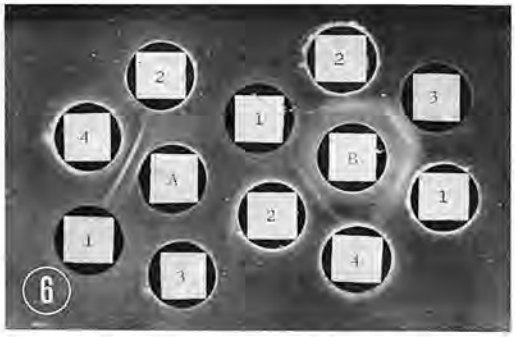


Figure 6 Immunodiffusion analysis of ultracentrifuge-purified JE vaccine.

A: Anti-ultracentrifuge-purified vaccine rabbit serum (RUV-9)
B: Anti-normal mouse brain rabbit serum (RMB-24)
1: Ultracentrifuge-purified vaccine (15 times concentration of the final vaccine product)
2: Supernatant from 30 per cent normal brain homogenate
3: Encephalitogenic basic protein from mouse brain (24 μg N/ml)
4: Normal mouse serum

6. *Side effects observed in people inoculated with ultracentrifuge-purified vaccine*

The protamine vaccine used previously usually gave severe local pain when injected. The alcohol-protamine vaccine is also said to cause this symptom to some extent. The ultracentrifuge-purified vaccine described in this paper, however, gives little or no pain. This difference might be due to the lower protein content of the latter, because it seems difficult at present to prepare an alcohol-protamine vaccine of high potency but containing less than 10 μg hot TCA-precipitable nitrogen. Table 4 shows the side effects observed in a group of subjects following the administration of the ultracentrifuge-purified vaccine. As shown in this table, this vaccine is one of the most favorable vaccines in practical use.

TABLE 4 *Side effects observed following administration of ultracentrifuge-purified JE vaccine**

	Number observed**	Per cent incidence
Local pain	3	0.4
Local pain on pressure	36	5.2
Local rash	21	3.1
Local swelling	0	0
Slight fever (less than 1°C rise)	3	0.4
High fever (more than 1°C rise)	0	0

* Administration : subcutaneous injection of 1.0 ml dose into upper arm on one side.
** Total number observed : 675 persons.

7. *Ultracentrifuge-purified vaccine prepared from the current strain, JaGAr#01*

For a long time the antigenic composition of JE virus in various strains was regarded to be homogeneous. However, as more strains were isolated from mosquitoes and from patients, their antigenic heterogeneity was revealed, but most of the current-isolated strains were shown to possess the similar antigenic structure (HALE and LEE, 1954; OGATA, 1959; KOBAYASHI, 1959). The Nakayama strain used for the present vaccine, was isolated by Kasahara and his coworkers in 1935 (KASAHARA *et al.*, 1936). It has a history of more than 100 passages in mouse brain, and is known to possess various antigenic differences from other currently-isolated strains (OGATA, 1959; KOBAYASHI, 1959). Oya and his coworkers reported that the JE patients and non-vaccinated adults in epidemic areas had the same levels of neutralizing antibody for both the Nakayama and the current strain, JaGAr#01 of JE virus (The JaGAr#01 strain was isolated from the mosquito *Culex tritaeniorhynchus* in 1959 (MATSUYAMA *et al.*, 1960)). Among the people immunized recently with the present Nakayama vaccine, however, some showed a remarkably higher level of neutralizing antibody against the homologous strain than against heterologous JaGAr#01 strains (OYA *et al.*, 1965; HAN *et al.*, 1967).

Hitherto, it has been regarded that the currently-isolated strains of JE virus do not give a good vaccine when prepared by a similar method to the present Nakayama vaccine. Thus, while the vaccine prepared from Nakayama strain-infected adult mouse brain could protect mice against intracerebral challenge from either the Nakayama or the currently isolated JaGAr#01 strain, vaccine prepared from the latter virus material failed to protect mice from either strain (OYA, 1966). Furthermore, it was reported that alcohol-protamine JaGAr#01 vaccine made from infected mouse brain gave a much lower antibody response when tested by the neutralization test by plaque reduction (OYA *et al.*, 1966).

In this respect, it was thought that the failures might be due to inadequate methods for preparing vaccine, and vaccine from adult mouse brains infected with the JaGAr#01 strain was prepared by the ultracentrifugation method. Table 5 shows the results of a mouse potency test by the direct challenge method. JaGAr#01 vaccine could protect immunized mice against challenge by the homologous strain, while it showed lower potency against challenge by the Nakayama strain. On the other hand, the Nakayama vaccine gave the same degrees of protection against challenge by either strain. Similar results were obtained by the neutralizing antibody test by plaque reduction, which has been proved to give a more accurate ED_{50} titer than the mouse potency test by direct challenge (OYA, 1966; OYA *et al.*, 1966; KUNITA *et al.*, 1966; NOBUTO *et al.*, 1966). Table 6 shows that both vaccines gave more effective antibody responses in immunized mice against the homologous virus strain than against the heterologous strain.

A preliminary field trial was carried out among the newly-employed technicians of the Kan-onji Institute using this JaGAr#01 vaccine. Although individuals, who were definitely known to have been immunized previously with JE (Nakayama) vaccine, were

TABLE 5 *Comparison of potencies of ultracentrifuge-purified vaccines prepared from the JaGAr #01 and Nakayama strains by the direct mouse challenge test*

Vaccine	Challenge virus									
	JaGAr#01 (13 mouse LD_{50})					Nakayama (51 mouse LD_{50})				
	Vaccine dilution					Vaccine dilution				
	4^0	4^1	4^2	4^3	ED_{50}	4^0	4^1	4^2	4^3	ED_{50}
JaGAr#01 vaccine**	0/10*	2/10	2/10	2/9	$4^{3.0}$	5/9	7/9	8/10	7/9	$4^{0.5}$
Nakayama vaccine***	0/7	2/8	2/10	2/10	$4^{2.3}$	0/10	0/9	4/9	5/8	$4^{2.6}$

* The numerator shows the numbers of deaths, and the denominator shows the total numbers in the group.
** Ultracentrifuge-purified JaGAr#01 vaccine.
*** Ultracentrifuge-purified Nakayama vaccine.

TABLE 6 *Comparison of potencies of ultracentrifuge-purified vaccines prepared from the JaGAr #01 and Nakayama strains by the neutralizing antibody test by plaque reduction*

Vaccine	Challenge virus									
	JaGAr#01 (85 PFU/plate)					Nakayama (112 PFU/plate)				
	Vaccine dilution					Vaccine dilution				
	4^1	4^2	4^3	4^4	ED_{50}	4^1	4^2	4^3	4^4	ED_{50}
JaGAr#01 vaccine**	2/9*	5/10	6/10	9/9	$4^{2.2}$	1/10	2/9	7/10	8/9	$4^{2.6}$
Nakayama vaccine***	1/9	5/8	8/10	9/10	$4^{2.0}$	0/9	1/8	4/8	4/10	$4^{3.6}$

* The numerator shows the numbers of plates showing less than 50 per cent of the number of plaques in the plate of control virus, and the denominator shows the total numbers of plates in the group.
** Ultracentrifuge-purified JaGAr#01 vaccine.
*** Ultracentrifuge-purified Nakayama vaccine.

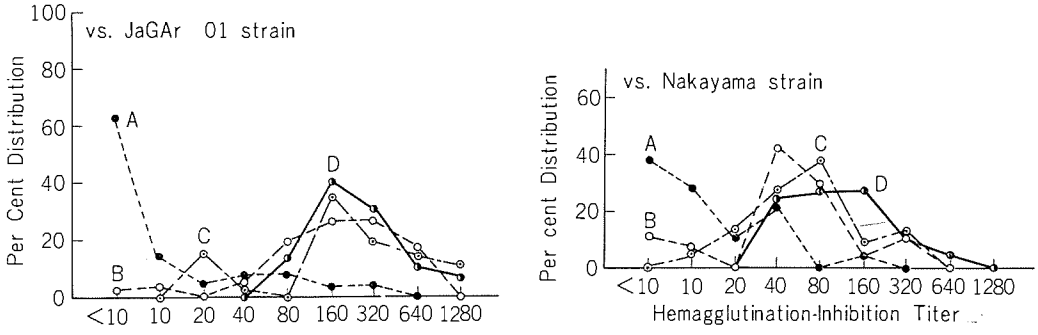


FIGURE 7 Distribution of hemagglutination inhibition titers against the JaGAr#01 and Nakayama strains of JE virus before and after immunization with ultracentrifuge-purified JaGAr#01 vaccine.
The vaccine was inoculated in doses of 1.0 ml on the 1st, 9th, and 30th days of the experiment.
Total number of immunized persons : 31.
A : Before immunization
B : 9th day after the first immunization
C : 21st day after the second immunization (30th day after the first immunization)
D : 17th day after the third immunization (47th day after the first immunization)

eliminated from this group, some individuals in the test group may have received JE vaccine previously but have forgotten about it, and some of them may have been abortively infected with JE before, as judged from the result obtained. Thirty-one persons were inoculated with this vaccine in a dose of 1.0 ml each on the 1st, 9th, and 30th day. Serum specimens were taken immediately before each inoculation and on the 47th day after the first immunization. Fig. 7 shows the hemagglutination-inhibition antibody responses. The antibody levels increased sharply against the homologous JaGAr#01 strain, and those to the heterologous Nakayama strain also increased to some extent.

DISCUSSION

Japanese encephalitis (JE) is one of the most important infectious diseases in Japan and neighboring countries. There are also many other diseases known to be caused by various arboviruses. For control of these diseases two methods are avialable: namely, eradication of arthropod vectors and immunization of individuals by vaccination.

In yellow fever the famous discovery of transmission of the causative agent through *Aedes aegypti* mosquitoes led to an anti-aegypti campaign which successfully eradicated yellow fever from various epidemic areas. In the case of JE, the complicated ecological behaviors of the vector, *Culex tritaeniorhynchus*, and the meteorological, agrico-sociological environment of this area, however, are making an anti-culex campaign very difficult.

Most arboviruses are known to multiply in mouse brain in a sufficiently high titer to permit preparation of an effective vaccines. The fear, however, of possible contamination of vacines with brain tissue substances causing allergic encephalomyelitis has prevented the practical use of this type of vaccine. Since formalin-inactivated vaccine from infected mouse brain was introduced in 1954 for the control of JE, extensive efforts have been made to raise the potency of the vaccine and more especially to eliminate possible encephalitogenic substances from it.

One of the authors reported that JE virus could be purified from the protamine extract of brain homogenate of infected mice by ultracentrifugation, and that the infectivity of the virus was sedimented coincidentally with its hemagglutinating activity. His purified preparation revealed homogeneous size of virus particles of 38 mμ in diameter with little contaminating materials under electron microscope. The sedimentation constant and the buoyant density of the virus were calculated as 180 ± 3 S and 1.24, respectively (FUKAI, 1962). Based on these experiments, we have studied on the purification of JE vaccine by ultracentrifugation on a large scale, resulting in the successful production of the purified vaccine for practical use as described in this paper.

The animal experiments shown in Table 3 indicate that ultracentrifuge-purified vaccine and even old type protamine vaccine did not cause allergic encephalomyelitis in monkeys even when inoculated at 50 times the normal concentration: while homogenates of normal rabbit spinal cord and of normal mouse brain caused clinical paralysis and/or histopathological lesions in the central nervous system. Similar results reported by the Committee on the Japanese Encephalitis Vaccine using cynomolgus monkeys and guinea pigs of the Hartley strain (EGASHIRA *et al.*, 1966; SHIRAKI, 1966) have reconfirmed the safety of the JE vaccine. Furthermore, immunodiffusion analysis, as shown in Fig. 2 reveals clearly that the purification steps employed in our procedure are very effective to remove the antigens which react with rabbit anti-normal mouse brain serum, and another set of experiments demonstrated the absence of possible contamination of the ultracentrifuge-purified vaccine preparation by encephalitogenic basic protein (cf. Fig. 6).

In practical measures, about 800 ml of the material can be processed at one time in a Hitachi ultracentrifuge, fitted with an RP-21

rotor. If the centrifuge is run 3 times a day, about 12 liters of the virus material can be handled in one centrifuge in 5 working days per week. Therefore, if 10 ultracentrifuges are available, 120 liters of the virus material, corresponding roughly to 60,000 to 70,000 mice, can be routinely managed a week.

Kanamitsu and his coworkers reported the results of a JE vaccine field trial in Wakkanai City, Hokkaido (KANAMITSU, 1966). They tested 2 types of purified JE vaccine on the boys and girls in junior high schools of the city who had never been exposed to JE virus. The results showed that ultracentrifuge-purified vaccine gave a better response than alcohol-protamine vaccine, irrespective of the inoculation frequency. All the pupils showed a good response after inoculation with the vaccine, and the serum neutralizing antibody titer 4 months after the first immunization was still high. Further surveys showed that the retention of antibody was fairly good even after one year, and all the pupils, who had received the vaccine one year previously, responded very well to a booster inoculation of this vaccine in a dose of 1.0 ml.

The same group of investigators have also carried out field surveys using the ultracentrifuge-purified vaccine preparation of the JaGAr #01 strain described in this paper, and obtained an excellent response from the pupils immunized (KANAMITSU *et al.*, 1968).

As mentioned before, however, it has long been believed that a good vaccine cannot be prepared from the JaGAr#01 strain by a similar method to that used for the present Nakayama vaccine. The preliminary results obtained on the ultracentrifuge-purified JaGAr#01 vaccine described above, open the way for reconsideration of the JaGAr#01 strain and preparation of better vaccine against Japanese encephalitis and related diseases.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the encouragement and advice of Drs. J. Kamahora, Y. Okuno, K. Toyoshima and T. Miwatani of the Research Institute for Microbial Diseases, Osaka University, Dr. M. Mutai of the Yakuruto Institute, Kunitachi, Tokyo, and Dr. S. Kano of the National Institute of Hygiene, Osaka. They also thank Mr. Oku and other staff of the Kan-onji Institute, the Research Foundation for Microcial Diseases of Osaka University for helpful discussion and assistance.

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