BIKEN JOURNAL Vol. 13, 25-38, 1970

JAPANESE ENCEPHALITIS PURIFIED VACCINE
II. PURITY OF THE MOUSE BRAIN VACCINE PURIFIED BY ULTRACENTRIFUGATION

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(Received December 2, 1969)

SUMMARY The quantitative micro-precipitin test using ^{125}I-labeled mouse or rabbit antibodies against Japanese encephalitis virus (JEV) showed that Japanese encephalitis vaccine, prepared from infected mouse brains and purified by ultracentrifugation, contained 10 to 15% viral antigen.

Immunodiffusion analysis with rabbit antiserum against encephalitogenic basic protein from mouse brain showed that the vaccine contained no antigen or if any, less than 0.3%. The passive cutaneous anaphylaxis (PCA) test also showed that the vaccine contained no encephalitogenic basic protein or if any, less than 0.035%.

Injection of the vaccine at a concentration of as high as 250 times that of the usual product, did not elicit allergic encephalomyelitis in guinea pigs of the Hartley strain, while injection of a homogenate of mouse brain caused definite paralysis and/or histopathological lesions of the central nervous system.

INTRODUCTION

The practical use of Japanese encephalitis vaccine purified from infected mouse brains started in 1966, and has led to a rapid increase in the number of vaccinated people in Japan and a rapid reduction in the incidence of this disease. The previous paper (Takaku et al., 1968) reported the method of purification of the vaccine, involving homogenization of brain from mice infected with Japanese encephalitis virus (JEV), Nakayama strain and treatment of the homogenate with protamine and active carbon and two cycles of ultracentrifugation. This method yields a very effective vaccine containing very little protein nitrogen. The purified vaccine is rather labile in phosphate-buffered saline, but can be stabilized by suspension in TC Medium 199 containing gelatin and phosphate buffer. Ultracentrifugation is also effective in purification of vaccine from mice infected with a recently-isolated strain

JaGAr #01 of JEV.

The purification procedure involving ultracentrifugation was found to eliminate most of the impurities derived from brain tissue (Takaku et al., 1968). However, there is still fear of possible contamination of the vaccine with brain tissue substances causing allergic encephalomyelitis. In this work, the quantitative micro-precipitin test using ^{125}I-labeled antibody to Japanese encephalitis viral antigen was employed to calculate the purity of the vaccine. It was found that the vaccine contained 10 to 15% antigen. Immunodiffusion analysis and passive cutaneous anaphylaxis (PCA) using antiserum against the encephalitogenic basic protein from mouse brain tissue also showed that this vaccine contained no antigen or if any, less than 0.3% (from data of immunodiffusion) or less than 0.035% (from PCA data). It was also shown that the vaccine at a concentration of as high as 250 times that of the usual product did not elicit allergic encephalomyelitis in guinea pigs of the Hartley strain.

MATERIALS AND METHODS

1. *Virus*

Japanese encephalitis virus (JEV), Nakayama (Nakayama-Yoken) strain, used for the preparation of mouse brain vaccine purified by ultracentrifugation (Takaku et al., 1968), was obtained from the National Institute of Health of Japan, and was maintained by alternate passage in mice and storage in liquid nitrogen. The current strain OH 0566, was isolated in the Virus Laboratory of the Osaka Public Health Institute, Osaka, Japan, from the brain of a patient who died of Japanese encephalitis, and was given to us by courtesy of Dr. Kunita. It was adapted to primary cynomolgus monkey kidney (MK) cells in this Institute by serial passages on the cells, on which it developed a cytopathic effect, and was used as the strain for preparation of inactivated vaccine from tissue culture (Yoshida et al., to be published). Virus titration was carried out by plaque counting on a chick embryo fibroblast culture by a modification of the method of Porterfield (1960).

2. *Vaccine from infected mouse brain purified by ultracentrifugation*

Infected mouse brain was homogenized, treated with protamine and active carbon and subjected to two cycles of ultracentrifugation as described previously (Takaku et al., 1968). Concentrated vaccine preparation was obtained from the final batches of vaccine from routine production line before dilution with stabilizing solution. Concentrated preparations of vaccine at various steps of purification were obtained either by ultrafiltration or by removal of medium containing low molecular solutes by swelling of calculated amounts of dry Sephadex G 25 in the samples.

3. *Encephalitogenic basic protein from mouse brain tissue*

Basic protein, which was found to be the causative antigen of allergic encephalomyelitis (Paterson, 1966) was extracted from normal mouse brains and partially purified by chromatographies on diethylaminoethyl (DEAE)-, and carboxymethyl (CM)-cellulose columns by the method of Kies (1965). When it was injected into the neck of guinea pigs of the Hartley strain at a dose of 1.5 μgN and 15 μgN in Freund's complete adjuvant, 60 to 90% of the animals developed definite paralysis of hind legs after 13 to 25 days.

4. *Antisera*

1) Mouse antiserum against JEV, Nakayama strain (MUV):

Mice, which survived intracerebral infection for the vaccine production, were further immunized with killed vaccine as follows. One week after the inoculation with the vaccine product they received, at weekly intervals 3 series of 3 intraperitoneal injection, at two-day intervals, of 0.5 ml of 7 times concentrated vaccine purified by ultracentrifugation (Takaku et al., 1968). Two weeks after the last injection, mice were bled. Their sera were pooled, and stored at -20C without any preservative.

2) Rabbit antiserum against JEV, strain OH 0566 (RTV):

Rabbits were inoculated intravenously with 4.0 ml of live JEV, strain OH 0566, at a concentration of about 2×10^8 plaque forming units (PFU)/ml which had been cultivated on primary MK cells (Yoshida et al., to be published). Two days after infection, they were injected intramuscularly with 10 ml of the same virus culture emulsified with an equal

volume of Freund's complete adjuvant containing 85% Drakeol, 15% Arlacel A, 2 mg/ml killed *Mycobacterium tuberculosis*, strain H37Rv, 5 mg/ml Viccilin and 20 mg/ml Kanamycin. They received 2 more intramuscular injections of 10 ml of the same culture with adjuvant at intervals of 18 days. Two months after the last injection, rabbits were bled 3 times (40 ml of blood each time by heart puncture) at intervals of 5 days and then bled to death 1 week later. Antisera were stored at −20 C without any preservative.

3) Rabbit antiserum against JEV (Nakayama) vaccine from infected mouse brain purified by ultracentrifugation (RUV):

Rabbits were immunized 4 times, at intervals of about one month, by intravenous injection of 2.0 ml of 4 times concentrated vaccine purified by ultracentrifugation and simultaneously by intramuscular injection of 10 ml of the same concentrated vaccine emulsified with an equal volume of Freund's complete adjuvant. Two weeks after the last inoculation, the rabbits were bled. Antisera were stored at −20 C without any preservative.

4) Rabbit antiserum against encephalitogenic basic protein from mouse brain (RBPM):

Rabbits were immunized by three injections into their footpads and necks at one month intervals of basic protein solution in 1.25% soluble carboxymethyl cellulose (Moroz et al., 1963) emulsified with an equal volume of Freund's complete adjuvant. The doses of antigen were 70 μgN in the first, 140 μgN in the second and 200 μgN in the third immunization. Some animals developed paralysis of the hind legs and some died during the course of immunization. Two weeks after the last immunization the rabbits were bled. Antisera were stored at −20 C without any preservative.

5) Goat antiserum against mouse serum proteins (GNMS), goat antiserum against rabbit serum proteins (GNRS) and rabbit antiserum against mouse serum proteins (RNMS):

These antisera were obtained by courtesy of Mr. M. Ichikawa and Mr. M. Hayashi of this Institute. These antisera gave 25 to 30 arcs against the mouse serum on an immunoelectrophoretic plate.

5. *Isolation of the γG globulin fractions from antisera*

The γG globulin fraction was obtained from mouse anti-JEV, Nakayama-antiserum (MUV) by a combination of ammonium sulfate precipitation and two cycles of gel filtration through a Sephadex G 150 column. If necessary, the fractions obtained during purification were concentrated by ultrafiltration.

The γG globulin fraction was also obtained from rabbit anti-JEV, OH 0566-antiserum (RTV) by ammonium sulfate precipitation followed by DEAE-cellulose chromatography.

These fractions were shown by immunoelectrophoresis to consist mainly of γG globulin and to be little contaminated with otber proteins. (Fig. 1)

6. *Radioactive labeling of γG globulin*

Mouse or rabbit γG globulin was labeled with ^{125}I by triiodide solution ($Na^{125}I$ was added in a solution of I_2 in KI) at 0 C for 4 hr in 0.2 M tris (hydroxymethyl) amino methane (Tris-HCl buffer, pH 8.5, by the method of Covelli and Wolff (1968). The molar ratio of iodine added to the globulin was 1.01 for the mouse antibody and 1.98 for the rabbit antibody in the experiment shown in Fig. 2. Iodinated protein was dialyzed against 100 volumes of phosphate-buffered saline, pH 8.0, at 4C for 48 hr with 4 changes of the dialysis fluid. After dialysis, the turbid solution was centrifuged at 3,000 rev/min for 30 min. The clear supernatant was used for the quantitative microprecipitin reaction. The molar ratio of iodine to γG globulin in these preparations was 0.21 for mouse antibody and 0.31 for rabbit antibody. Both the mouse and rabbit γG globulin preparations contained less than 2% of the total iodine as free iodine as judged by the radioactivity in the supernatant after precipitation of the preparations with trichloroacetic acid.

The antibody and immune precipitates were counted in a Toshiba Flow Counter Scaler, Model UDS-24204 with a No. 4 Gain Select at 1 KV of HI voltage.

In the region of antibody excess and the equivalence zone, all the antigen, JEV in this experiment, should be precipitated by antibody. The amount of antibody (Ab) precipitated can be calculated from the radioactivity precipitated. Therefore, the purity of the vaccine preparation can be calculated as following:

$$\text{Purity} = \frac{\text{Total N ppted}-\text{Ab N ppted}}{\text{Vaccine N added}}$$

Where: Ab N ppted = KC

K: a conversion constant for the ^{125}I-labeled antibody used

C: radioactivity of the precipitate minus background radioactivity

FIGURE 1. *Immunoelectrophoretic pattern of γG globulin fractions from mouse and rabbit anti-JEV-antisera.*
a) Upper pattern: mouse γG globulin fraction
Lower pattern: normal mouse serum
Goat anti-normal mouse serum-antiserum was placed in the trough.
b) Upper pattern: rabbit γG globulin fraction
Lower pattern: normal rabbit serum
Goat anti-normal rabbit serum-antiserum was placed in the trough.

7. *Immunoelectrophoresis*

This was carried out according to a modification of the method of Scheidegger (1955) using 1% agarose in veronal-acetate buffer, pH 8.6, $\mu=0.05$.

8. *Immunodiffusion analysis (Ouchterlony technique)*

This was carried out in 0.7% agarose in phosphate-buffered saline, pH 7.2.

9. *Animals*

Guinea pigs of the Hartley strain were purchased from Shizuoka Experimental Animals Corp., Hamamatsu, Shizuoka. Mice and rabbit were supplied from local dealers.

10. *Nitrogen determination*

Nitrogen was determined by the Kjeldahl-Nessler method of Yokoi and Akashi (1955).

11. *Chemical*

Diethylaminoethyl (DEAE)-cellulose was obtained from Brown Co., Berlin, N. H. Carboxymethyl (CM)-cellulose (Serva) was from Gallard-Schlesinger Chemical Mfg. Corp., Long Island, N. Y. Soluble CM-cellulose was obtained from Nichirin Chemicals Industry Co., Osaka. Sephadex G 50 and G 25 were obtained from Pharmacia, Uppsala, Sweden. $Na^{125}I$, free of carrier and reducing agent, was purchased from Daiichi Chemicals Co., Tokyo.

RESULTS

1. *Quantitative micro-precipitin test of the vaccine against ^{125}I-labeled anti-JEV- antibody*

For estimating the purity, i.e., viral antigen

content of vaccine purified by ultracentrifugation, quantitative micro-precipitin tests were carried out using ^{125}I-labeled anti-JEV-antibodies. Two kinds of antibodies were used: a) mouse antibody against mouse brain (Nakayama) vaccine purified by ultracentrifugation (MUV) and b) rabbit antibody against the currently-isolated strain, OH 0566, of JEV cultured in primary MK cells (RTV). Neither antibody showed cross reaction against either the supernatant from a homogenate of mouse brain or normal mouse serum.

Various amounts of antigen in 1.8 ml of M/100 trisodium ethylenediamine tetraacetate (EDTA) in phosphate-buffered saline (0.15 M NaCl in 0.02 M phosphate buffer), pH 8.0 were incubated in 13 × 78 mm conical tubes with 50 μl of ^{125}I-labeled γG globulin fraction of mouse anti-JEV-antiserum at 37 C for 60 min and then at 4 C overnight. With rabbit antibody, antigen in 2.6 ml of phosphate-buffered saline containing EDTA was incubated similarly with 400 μl of labeled antibody. Controls without antigen or antibody were also set up. Then the reaction mixtures were centrifuged and the supernatants were tested for excess antigen and

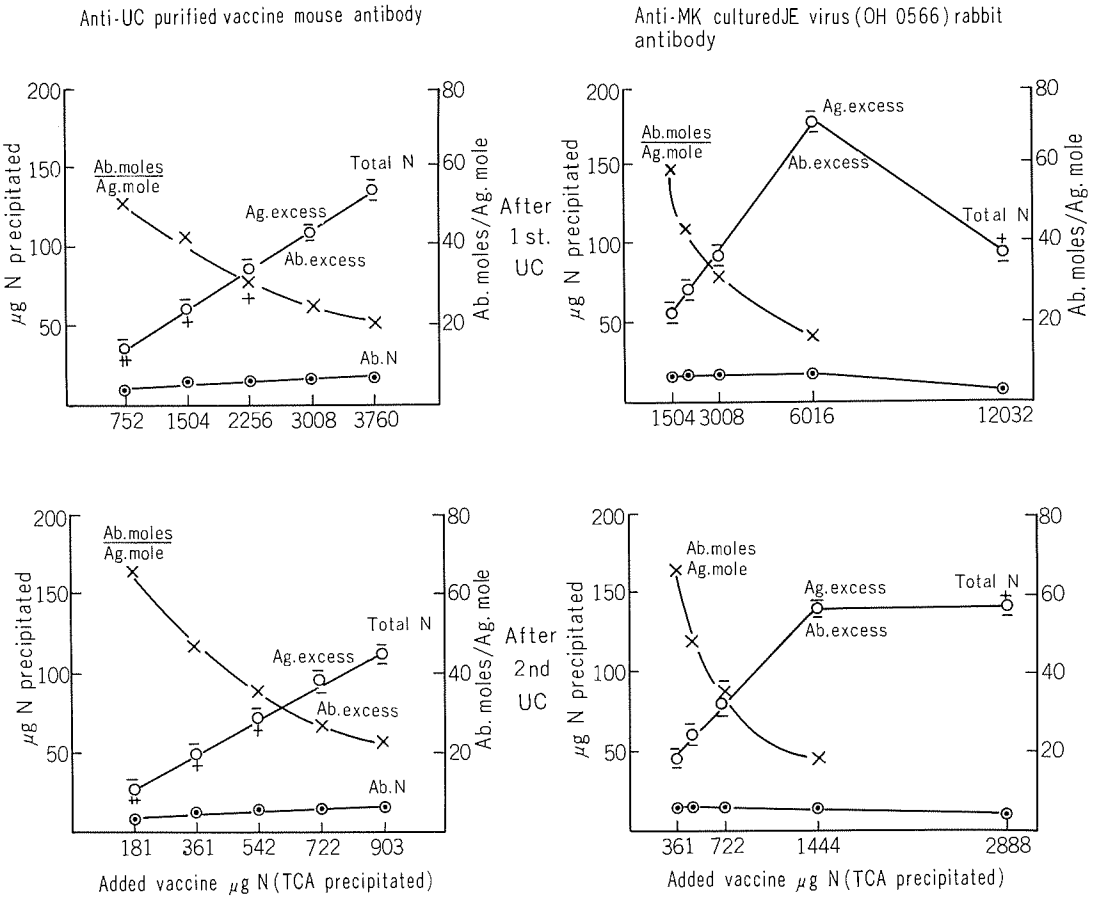

FIGURE 2. *Quatnitative precipitin reaction of vaccine purified by ultracentrifugation.*
Antigens: pellet and bottom 1/20 of supernatant of 1st (upper) and 2nd (lower) ultracentrifugation
Antibodies: mouse antibody against (Nakayama) vaccine from infected mouse brain purified by ultracentrifugation (left) and rabbit antibody against JEV, strain OH 0566 cultured on MK cells (right)

antibody. The precipitates were washed three times with 2.0 ml of cold phosphate-buffered saline, pH 8.0 and then dissolved in 3.0 ml of 0.1 N NaOH. Two ml of each solution were taken for nitrogen determination, and 0.5 ml was used for measurement of radioactivity.

The experiment shown in Fig. 2 was to compare mouse and rabbit antibodies and to estimate the effect of two cycles of ultracentrifugation in the purification of vaccine (Takaku et al., 1968). The antigens used were the

TABLE 1. *Quantitative micro-precipitin reaction of purified vaccine.*

Anti-UC purified vaccine mouse antibody

Concentrated UC-purified vaccine N	Total N precipitated	Antibody N calculated from c.p.m.	Antigen N	Ab. molecules / Ag. molecule in precipitated complex	Ab. N / Ag. N in precipitated complex	Supernatant test: for excess antibody	Supernatant test: for excess antigen	Purity of vaccine (%)
			After 1st UC					
μg	μg	μg	μg					
752	34.7	9.1	25.6	51.6	0.342	++	—	3.4
1504	59.0	12.9	46.1	41.8	0.280	+	—	3.1
2256	84.7	14.4	70.3	30.6	0.205	+	—	3.1
3008	107.2	15.2	92.0	24.6	0.165	—	—	3.1
3760	132.9	16.2	116.7	20.8	0.139	—	—	3.1
			After 2nd UC					
181	27.1	8.3	18.8	65.9	0.442	++	—	10.0
361	49.3	11.8	37.5	47.0	0.315	+	—	10.4
543	70.8	13.5	57.3	35.2	0.236	+	—	10.6
722	94.4	14.2	80.2	26.4	0.177	—	—	11.1
903	110.7	14.6	96.1	22.7	0.152	—	—	10.6

Anti-MK cultured JE virus (OH 0566) rabbit antibody

Concentrated UC-purified vaccine N	Total N precipitated	Antibody N calculated from c.p.m.	Antigen N	Ab. molecules / Ag. molecule in precipitated complex	Ab. N / Ag. N in precipitated complex	Supernatant test: for excess antibody	Supernatant test: for excess antigen	Purity of vaccine (%)
			After 1st UC					
μg	μg	μg	μg					
1504	55.8	15.7	40.1	58.3	0.391	—	—	2.7
2010	70.3	15.8	54.5	43.3	0.290	—	—	2.7
3008	91.4	15.8	75.6	31.2	0.209	—	—	2.5
6016	175.2	17.0	158.2	16.0	0.107	—	—	2.6
12032	94.0	6.7	—	—	—	—	+	
			After 2nd UC					
361	45.1	13.8	31.3	65.8	0.441	—	—	8.7
481	60.4	14.7	45.7	48.1	0.322	—	—	9.5
722	80.5	15.2	65.3	34.8	0.233	—	—	9.1
1444	139.7	15.4	124.3	18.5	0.124	—	—	8.6
2888	141.9	11.2	—	—	—	—	+	

pellets plus the bottom 1/20 of the supernatants from the first and second ultracentrifugation cycles of a single batch of inactivated protamine extract after treatment with active carbon (Takaku et al., 1968). The precipitin curves for mouse antibody in this experiment cover only the region from antibody excess to the equivalence zone as judged by the supernatant test. However, the curves for rabbit antibody extend to the antigen excess region.

Table 1 shows the actual values obtained and the purities of these vaccine preparations calculated from the results. In this case, the mouse γG globulin preparation contained 3.94 mgN/ml and 532 cpm/μgN of antibody and the rabbit preparation contained 1.51 mgN/ml and 1,640 cpm/μgN antibody. With both mouse and rabbit antibodies the values obtained for the purities of these vaccine preparations coincide well.

From several similar experiments, it was concluded that the final preparations of the mouse brain vaccine purified by ultracentrifugation contained 10 to 15% viral antigen.

2. *Immunodiffusion analysis for detection of possible contamination of the vaccine with encephalitogenic basic protein*

The preceding experiments showed that after ultracentrifugation the vaccine contained 85 to 90% impurity. There was a fear of possible contamination of the vaccine with brain tissue substances causing allergic encephalomyelitis like in the many tragic cases caused by rabies vaccine. Recently, a basic protein located on the myelin sheath of the central nervous system of various mammals has been shown to produce allergic encephalomyelitis in experimental animals (Robez-Einstein et al., 1962; Rauch and Raffel, 1964; Kies, 1965; Paterson, 1966).

As shown previously (Takaku et al., 1968), the antigens reacting with antiserum against normal mouse brain homogenate are eliminated as purification proceeds. Anti-normal mouse brain homogenate-antiserum sometimes developed a few faint arcs against vaccine purified by ultracentrifugation and concentrated 15 times, but none of these arcs either fused into, or formed spurs with the arc formed between the antiserum and the encephalitogenic basic protein prepared form mouse brain tissue (Takaku et al., 1968). These results show that there is little possibility of contamination of the vaccine with encephalitogenic antigen. However, this possibility was examined further using both the antiserum against the encephalitogenic basic protein, purified by the method of Kies (1965), and more concentrated vaccine preparation.

Fig. 3 shows results of immunodiffusion analysis of preparations of vaccine at various

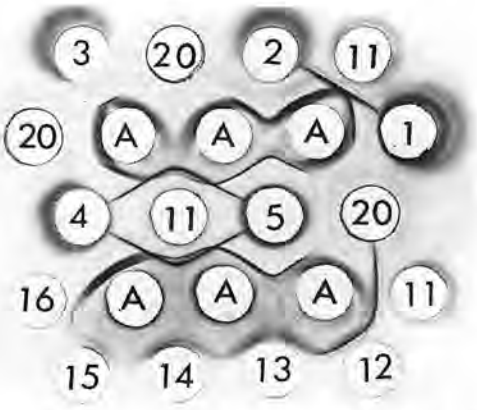

FIGURE 3. *Immunodiffusion analysis of samples at various purification steps against rabbit anti-encephalitogenic basic protein antiserum.*
A: Rabbit anti-encephalitogenic basic protein-antiserum (RBPM-2).
1: Supernatant of 60% normal mouse brain homogenate.
*2: * Protamine extract.*
3: Supernatant after active carbon treatment.
*4: * Supernatant from ultracentrifugation.*
*5: * Pellet and bottom 1/20 of the supernatant from ultracentrifugation.*
11–16: Encephalitogenic basic protein from mouse brain (11: 160 μgN/ml, 12: 80 μgN/ml, 13: 40 μgN/ml, 14: 20 μgN/ml, 15: 10 μgN/ml and 16: 5 μgN/ml).
20: Normal mouse serum.
** The antigens used were from a single batch of protamine extract at various stages of purification, and their concentrations were adjusted to 200 times that of the final vaccine product.*

steps of purification and of various concentrations of encephalitogenic basic protein against rabbit antiserum against the basic protein.

The supernatant from the 60% normal mouse brain homogenate (Well No. 1) and normal mouse serum (Wells No. 20) were not concentrated but the concentrations of the other samples at various purification steps were adjusted to 260 times that of the final vaccine product. Serial dilutions of the basic protein isolated from mouse brain were placed in wells Nos. 11 to 16.

Fig. 3 shows that a precipitin arc formed between the antiserum used (Well A) and the concentrated vaccine (Well No. 5) and this arc fused with the arc formed by the encephalitogenic basic protein (Well No. 11) or the arc formed by normal mouse serum (Well No. 20). However, another arc formed between the antiserum and the basic protein and this neither fused into, nor formed a spur with the arc formed by the concentrated vaccine. This main precipitin arc was very clearly formed by the concentrated vaccine. This main precipitin arc was very clearly formed against the encephalitogenic basic protein, even at a concentration as low as 5 μgN/ml (Well No. 16). The concentrated protamine extract (Well No. 2) also formed a faint arc. This arc formed a spur with the main precipitin arc by the basic protein. A similar faint arc was also observed between the antiserum and the supernatant after treatment with active carbon.

The arcs, which formed and fused with each other between the antiserum and the preparations at various purification steps or normal mouse serum, were not due to encephalitogenic antigen but to the antibody against an impurity in the preparation of basic protein isolated by Kies' method, in this hyper-immune antiserum. It seems that the concentrated vaccine contains no encephalitogenic basic protein. It does not even contain as little as less than 5 μgN/ml, because, if the vaccine contained a small amount of encephalitogen too low to develop a visible precipitin arc, the main arc formed by this encephalitogen (Well No. 11) would be bent toward the antiserum well near the well containing concentrated vaccine (Well No. 5).

On the other hand the faint arc formed by the concentrated protamine extract and by the concentrated supernatant after treatment with active carbon show that these preparations may contain a little encephalitogen. Therefore, this immunodiffusion analysis clearly shows the necessity and value of ultracentrifugation in purification of the vaccine.

This assay shows that vaccine purified by ultracentrifugation and concentrated 260 times (Well No. 5) (1,495 μgN/ml in this experiment) contains no basic protein or if any, less than 5 μgN. Therefore, mouse brain vaccine purified by ultracentrifugation contains no encephalitogenic basic protein or if any, less than 0.3%.

To study the relationship between the precipitin arcs shown in Fig. 3, the antiserum was absorbed with insolubilized normal mouse serum proteins.

Fifteen ml of normal mouse serum were insolubilized by treatment with glutaraldehyde according to the method of Avrameas and Ternynck (1969). The insolubilized proteins were washed three times with phosphate-buffered saline, pH 7.4. Volumes of 4 ml of the antiserum used in the preceding experiment were absorbed on packed gels at 4 C overnight. Then the insoluble gels were removed by centrifugation and immunodiffusion analysis was carried out as in the preceding experiment.

As shown in Fig. 4 this absorbed antiserum (A') still formed a precipitin arc against encephalitogenic basic protein but no longer formed one against the vaccine. The faint arc formed between antiserum and the concentrated protamine extract (Well No. 2) was still seen, even after absorption of the antiserum with insolubilized normal serum proteins, and it formed a spur with the arc against the basic protein.

3. *Passive cutaneous anaphylaxis (PCA) test for encephalitogenic basic protein in the vaccine*

To test the possibility of contamination of

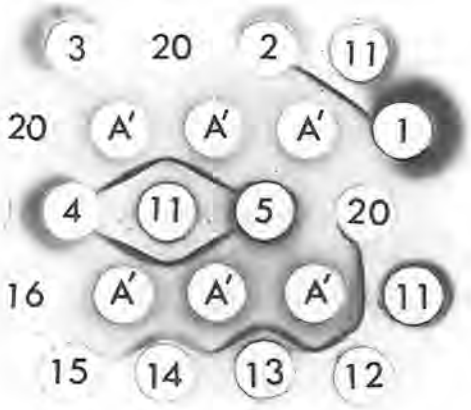

FIGURE 4. *Immunodiffusion analysis of samples at various purification steps against rabbit anti-encephalitogenic basic protein antiserum.*

The same antigens were used as for Fig. 3. The antiserum in Wells A' was also the same as in Fig. 3 but had been absorbed with insolubilized normal mouse serum proteins, as described in the text.

the vaccine with encephalitogenic basic protein further a passive cutaneous anaphylaxis (PCA) test was carried out.

Guinea pigs of the Hartley strain, weighed 400 to 450 g, were sensitized by intracutaneous injection of 0.1 ml doses of various dilutions of antiserum, usually in 4 rows of 6 spots on the back of a single animal. Six hours later, antigens in 1.0 ml of 3.0 mg/ml Evans blue solution were injected intravenously. Twenty minutes later the animals were sacrificed, and the sizes of the blue spots were measured from the under-surface of the skin. Absorption of antisera with insolubilized normal mouse serum proteins was carried out as described in the preceding paragraph.

As shown in Table 2, the encephalitogenic basic protein preparation elicited positive reactions against various dilutions of homologous antisera even at a concentration as low as 0.3 μgN/ml. This antigen preparation, when injected at a higher concentration, sometimes gave weak reaction against rabbit antiserum against vaccine purified by ultracentrifugation. However, the antibodies reacting with basic protein preparation were removed from this antiserum by absorption of the serum with insolubilized normal mouse serum proteins. Antibodies reacting with concentrated vaccine purified by ultracentrifugation or normal mouse serum could also be removed from rabbit anti-basic protein-antiserum by a similar absorption procedure.

Concentrated vaccine purified by ultracentrifugation prepared from infected mouse brains gave a positive reaction with homologous rabbit antiserum and also with rabbit anti-basic protein-antiserum and rabbit anti-normal mouse serum-antiserum, but gave negative reactions with mouse homologous antiserum and rabbit anti-tissue-cultured JEV (OH 0566)-antiserum. The sensitizing activities of rabbit homologous antiserum and of rabbit anti-basic protein-antiserum to concentrated vaccine were removed by absorption of the antisera with insolubilized normal mouse serum proteins. On the other hand, normal mouse serum used as an antigen also gave positive reactions with rabbit or goat homologous antiserum, rabbit anti-basic protein-antiserum and rabbit antiserum against vaccine puri fied by ultracentrifugation, but not with either of the latter antisera after their absorption with insolubilized normal mouse serum proteins.

Thus, after removing a common antibody or antibodies to normal mouse serum proteins from these antisera by the absorption technique, it was demonstrated that the encephalitogenic basic protein antigen only reacted with homologous antisera, and not with anti-vaccine-antiserum. On the other hand, concentrated vaccine purified by ultracentrifugation did not give a positive reaction even against homologous rabbit antiserum absorbed with insolubilized mouse serum proteins. It is uncertain why the vaccine did not give a positive reaction with mouse homologous antiserum, absorbed rabbit homologous antiserum or rabbit anti-tissue -cultured JEV-antiserum. However, if the concentrated vaccine preparation contained encephalitogenic basic protein as a contaminant at a concent-

TABLE 2. *PCA Test for Encephalitogenic Basic Protein and Vaccine Purified by Ultracentrifugation*

Antigen		Encephalitogenic Basic Protein from Mouse Brains (μgN/ml)							
Antiserum	Dilution	30.3	30.3	1.5	1.5	0.6	0.6	0.3	0.3
RBPM-3[a]	2×10	NT[c]	NT	15×14	15×15	15×18	14×13	19×16	16×18
	$2^2\times10$	NT	NT	12×10	12×10	11×10	13×12	15×16	10×10
	$2^3\times10$	NT	NT	7×11	15×13	10×9	10×11	15×15	8×8
	$2^4\times10$	NT	NT	11×11	11×13	9×9	6×4	12×16	8×7
	$2^5\times10$	NT	NT	8×9	8×9	7×6	6×7	—	—
RUV-12 (abs.)[b]	2×10	NT	NT	15×15	13×15	15×17	14×12	18×16	15×17
	$2^2\times10$	NT	NT	13×10	11×12	12×10	12×12	15×15	12×11
	$2^3\times10$	NT	NT	12×10	11×10	9×9	8×7	15×15	10×8
	$2^4\times10$	NT	NT	11×10	10×8	8×6	6×5	11×10	8×8
RBPM-6	5	21×21	20×21	NT	NT	NT	NT	NT	NT
	2×10	17×21	21×22	13×12	14×15	14×12	12×11	15×18	10×13
	2×10	21×15	17×18	10×11	11×10	15×13	10×8	15×17	9×9
	$2^2\times10$	18×15	15×15	7×8	9×8	6×8	4×5	10×12	8×7
RBPM-6 (abs.)[b]	5	20×18	14×14	14×14	13×11	15×14	13×14	16×16	12×12
	10	15×13	14×13	11×11	12×10	13×12	13×13	12×13	11×10
	2×10	12×12	11×14	10×11	10×8	15×14	11×10	11×10	9×9
	$2^2\times10$	10×10	10×9	NT	NT	NT	NT	NT	NT
MUV-15	1	—	—	NT	NT	NT	NT	NT	NT
	2	NT	NT	NT	NT	NT	NT	NT	NT
	2^2	NT	NT	—	—	—	—	—	—
RUV-12	1	5×5	—	NT	NT	NT	NT	NT	NT
	2	—	—	NT	NT	NT	NT	NT	NT
	2^2	—	—	NT	NT	NT	NT	NT	NT
	2^3	—	—	NT	NT	NT	NT	NT	NT
RUV-12 (abs.)[b]	1	—	—	—	—	—	—	—	—
	2	—	—	NT	NT	NT	NT	NT	NT
	2^2	—	—	NT	NT	NT	NT	NT	NT
RTV-8	1	—	—	NT	NT	NT	NT	NT	NT
	2	—	—	NT	NT	NT	NT	NT	NT
	2^2	—	—	NT	NT	NT	NT	NT	NT
RNMS-14	1	—	—	—	—	—	—	—	—
	2	—	—	—	—	—	—	—	—
	2^2	—	—	NT	NT	NT	NT	NT	NT
	2^3	NT	NT	NT	NT	NT	NT	NT	NT
GNMS-2	1	NT	NT	—	—	—	—	—	—
NRS	1	—	—	NT	NT	—	—	—	—
	10	NT	NT	—	—	NT	NT	NT	NT
NMS	1	—	—	NT	NT	NT	NT	NT	NT
	2^2	NT	NT	—	—	—	—	—	—
NGS	1	NT	NT	—	—	—	—	—	—
PBS		—	—	—	—	—	—	—	—

[a] RBPM : rabbit antiserum against encephalitogenic basic protein isolated from mouse brains ; MUV : mouse against mouse brain (Nakayama) vaccine purified by ultracentrifugation ; RTV : rabbit antiserum against tissue- against normal mouse serum ; NRS : normal rabbit serum ; NMS : normal mouse serum ; NGS : normal goat

[b] Antiserum absorbed with insolubilized normal mouse serum proteins.

[c] Figures combined with a × represent the cross diameters of the blue spot measured on the under-surface of

		Vaccine (times concentrated)						Normal Mouse Serum	
0.03	0.03	260	260	130	130	65	65	1/1	1/1
—	—	NT	NT	NT	NT	NT	NT	—	—
—	—	NT	NT	NT	NT	NT	NT	NT	NT
—	—	NT	NT	NT	NT	NT	NT	NT	NT
—	—	NT	NT	NT	NT	NT	NT	NT	NT
—	—	NT	NT	NT	NT	NT	NT	NT	NT
—	—	NT	NT	NT	NT	NT	TN	NT	NT
—	—	NT	NT	NT	NT	NT	NT	NT	NT
—	—	NT	NT	NT	NT	NT	NT	NT	NT
—	—	NT	NT	NT	NT	NT	NT	NT	NT
NT	NT	17×13	13×15	14×14	11× 9	—	—	11×11	12×10
—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—
—	—	NT	NT	NT	NT	NT	NT	—	—
—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—
NT	NT	NT	NT	NT	NT	NT	NT	—	—
NT	NT	—	—	—	—	—	—	NT	NT
NT	NT	—	—	—	—	—	—	NT	NT
—	—	—	—	—	—	—	—	NT	NT
NT	NT	18×15	17×15	14×15	16×15	12×11	12× 8	23×22	23×23
NT	NT	14×15	14×14	17×15	12×11	8×10	7× 7	20×18	21×20
NT	NT	12×14	12×13	5× 4	6× 6	4× 6	—	15×16	16×18
NT	NT	—	—	—	—	—	—	14×13	14×15
—	—	—	—	—	—	—	—	—	—
NT	NT	—	—	—	—	—	—	—	—
NT	NT	—	—	—	—	—	—	—	—
NT	NT	—	—	—	—	—	—	NT	NT
NT	NT	—	—	—	—	—	—	NT	NT
NT	NT	—	—	—	—	—	—	NT	NT
—	—	17×19	—	16×15	—	—	—	23×21	23×23
—	—	—	—	—	—	—	—	21×17	22×20
NT	NT	NT	NT	NT	NT	NT	NT	17×20	21×21
NT	NT	NT	NT	NT	NT	NT	NT	14×15	17×20
—	—	NT	NT	NT	NT	NT	NT	—	16×17
—	—	—	—	—	—	—	—	—	—
NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
NT	NT	—	—	—	—	—	—	NT	NT
—	—	NT	NT	NT	NT	NT	NT	NT	NT
—	—	NT	NT	NT	NT	NT	NT	—	—
—	—	—	—	—	—	—	—	—	—

antiserum against mouse brain (Nakayama) vaccine purified by ultracentrifugation; RUV: rabbit antiserum cultured JEV (OH 0566); RNMS: rabbit antiserum against normal mouse serum; GNMS: goat antiserum serum; PBS: phosphate-buffered saline, pH 7.2.

the skin. —: negative reaction; NT: not tested.

ration of as low as 0.3 μgN/ml, it should have been detected using rabbit antiserum against this antigen, which had been absorbed with insolubilized normal mouse serum proteins. Therefore, these results indicated that the 260 times concentrated vaccine (856 μgN/ml in this experiment) contained no encephalitogenic basic protein or if any, less than 0.3/856 or 0.035%.

4. *Experimental allergic encephalomyelitis in guinea pigs caused by various preparations from the central nervous system of mice*

The previous paper (Takaku et al., 1968) showed that neither concentrated vaccine purified by ultracentrifugation nor concentrated vaccine after protamine treatment, which was used clinically before introduction of vaccine purified by ultracentrifugation, elicited allergic encephalomyelitis in cynomolgus monkeys.

Experimental allergic encephalomyelitis induced by various preparations from the central nervous system of mice and by vaccines was reexamined using guinea pigs of the Hartley strain, which are known to be very sensitive to experimental allergic encephalomyelitis. The concentrated vaccine was obtained from a final batch of a routine product without dilution with stabilizing medium, as described in Materials and methods, instead of being concentrated by lyophilization used in the previous experiment (Takaku et al., 1968), which might result in degeneration or denaturation of possibly contaminated encephalitogen.

Antigens were injected into the two foot-pads of guinea pigs with Freund's complete adjuvant. The animals were examined for development of paralysis or death for 5 weeks after the injection. All animals which died and all those which survived the experimental period were examined histopathologically by study of preparations stained with hematoxylin-eosin.

As shown in Table 3, animals receiving vaccine purified by ultracentrifugation and concentrated 250 times did not develop paralysis. These animals were killed after 35 days and compared with control animals. No lesions of allergic encephalomyelitis were found in them in the cerebrum, cellebellum, pons, medulla oblongata or spinal cord. Fifty times

TABLE 3. *Induction of allergic encephalomyelitis (AE) in guinea pigs of the Hartley strain, with concentrated vaccine and various preparations of mouse central nervous system.*

Antigen[a]	Sensitizing dose/animal (mg Hot-TCA-precipitable N)	No. of animal sensitized	No. of animals with	
			Signs of AE	Lesion of AE
250× Concentrated UC-purified vaccine	0.4	11	0	0
50× Concentrated protamine vaccine	2.7	11	0	0
50% Normal mouse brain homogenate (MB)	2.3	11	4	8
2× Concentrated supernatant of MB	3.0	11	0	0
Protamine precipitate from MB	3.2	11	0	0
14.6× Concentrated protamine extract from MB	2.3	11	0	3
50% Mouse spinal cord homogenate	2.3	11	0	3
Adjuvant control[b]	—	8	0	0
Unsensitized control	—	5	0	0

[a] Antigen was mixed with an equal volume of adjuvant containing 85% Drakeol No. 6, 15% Arlacel A, 2 mg/ml killed *Mycobacterium tuberculosis*, strain H37RV, 5 mg/ml Viccilin, 20 mg/ml Kanamycin. It was injected into two foot pads.
[b] Phosphate-buffered saline was injected with adjuvant.
[c] Received no injection.

concentrated vaccine after protamine treatment also caused no positive clinical signs or histopathological lesions.

On the other hand, some animals which had received 50% mouse brain homogenate developed definite paralysis of their hind legs while others developed lesions, mainly appearing as perivascular infiltration of round cells, in their central nervous system even though they developed no clinical signs. Similar lesions were observed in animals receiving a mouse spinal cord homogenate or the supernatant (extract) or precipitate of a mouse brain homogenate after protamine treatment.

DISCUSSION

Since 1954 formalin-inactivated vaccine prepared from infected mouse brains has been used clinically as a vaccine against Japanese encephalitis. Year by year the potency of the vaccine has been increased and impurities eliminated. In 1966 purified vaccines, including our preparation purified by ultracentrifugation (Takaku et al., 1968) were introduced into practice. Since then the number of cases and deaths by this disease has rapidly declined. However, there is still the fear that vaccine prepared from brain tissue may cause tragic cases of allergic encephalomyelitis.

The quantitative micro-precipitin test was used to estimate the purity of the vaccine, and showed that after purification by ultracentrifugation the vaccine contained 10 to 15% viral antigen. This prompted us to reexamine the possibility of contamination of the vaccine with brain tissue substances causing allergic encephalomyelitis. It is well established that a basic protein or peptide located on the myelin sheath of the central nervous system is the antigen causing this disease (Robez-Einstein et al., 1962; Rauch and Raffel, 1964; Paterson, 1966). Both immunodiffusion analysis and the PCA test using antibody against this antigen showed that the vaccine contained no encephalitogen or if any, less than 0.3% (from immunodiffusion analysis) or less than 0.035% (from PCA). Experiments on the development of allergic encephalomyelitis using concentrated vaccine as well as various preparations of mouse brain tissue also showed that the vaccine, at a concentration of as high as 250 times that of the usual commercial product, did not elicit allergic encephalomyelitis in guinea pigs of the Hartley strain, while a brain tissue homogenate or other preparations of the central nervous system of mice caused distinct paralysis of the hind legs and/or histopathological lesions in the central nervous system of animals. Immunodiffusion analysis also showed the effectiveness of the ultracentrifugation method in purification of the vaccine. Both the concentrated protamine extract and the supernatant after treatment with active carbon still formed a faint precipitin arc against anti-encephalitogenic basic protein-antiserum, even after absorption of the antiserum with insolubilized mouse serum proteins. However, the samples from later purification steps gave no precipitin arc against the absorbed antiserum, which forms an arc against the basic protein. This also demonstrated the necessity and value of ultracentrifugation in purification of the vaccine.

The quantitative micro-precipitin test using ^{125}I-labeled antibody showed that this vaccine contained 10 to 15% JEV antigen. It was shown that JEV is a spherical particle of 38 mμ diameter with a buoyant density of 1.24 (Fukai in Takaku et al., 1968). From these values, assuming that the nitrogen content of JEV is 16%, a single particle of JEV should weigh 3.56×10^{-11} μg or 5.69×10^{-12} μgN. Vaccine purified by ultracentrifugation contains an average of 3 μg of nitrogen per ml precipitated by hot trichloroacetic acid, and 2 to 5×10^{8} PFU on chick fibroblasts per ml when prepared by the "live virus procedure" (Takaku et al., 1968) without formalin inactivation. From these values the vaccine should possess 5.3 to 8.0×10^{10} JEV particles per ml, if all the viral antigen precipited with the specific antibody can be counted as viral particles. Therefore, the ratio of viral particles to

infectious units on chick fibroblasts is calculated as 100 to 400. When JEV preparations are measured by 50% lethal doses (LD_{50}) for suckling mice infected intracerebrally, 4 to 5 times higher values are usually obtained as compared to the PFU values on chick fibroblasts. Then the ratio of viral particles to the LD_{50} is calculated as 20 to 100. These values do not mean that JEV is inactivated during the " live virus procedure " of purification, in which the final yield in PFU has routinely been observed to be more than 60% of that of a brain homogenate, but rather indicates that the brain tissue of infected mice can synthesize more non-infective virus particles than infective ones.

The results of quantitative micro-precipitin analysis described here also suggest that this method may be used without using labeled antibody. If a fairly wide range of equivalence zone is obtained as in the case described here, increase in the precipitate should be due to increase in the amount of antigen precipitated within the equivalence zone. Therefore, in such cases, the purity can be readily calculated.

ACKNOWLEDGEMENTS

The authors wish to express their sincere appreciation to Dr. Y. Okuno, Dr. J. Kamahora and Dr. S. Tanabe of the Research Institute for Microbial Diseases, Osaka University, and Dr. N. Kunita of Osaka Public Health Institute for their encouragement and advice through this work. They also gratefully acknowledge the helpful discussion and assistance of Mr. J. Oku, Mr. M. Ichikawa, Mr. I. Yoshida, Mr. T. Kan, Mr. M. Hayashi, Mr. H. Goda, Mr. H. Miyake and other members of the staff of the Kanonji Institute, the Research Foundation for Microbial Diseases of Osaka University.

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