Acta Medica Okayama

Volume 24, *Issue* 6 1970 *Article* 4

DECEMBER 1970

Vaccination with complete adjuvant-added inactivated virus vaccine of Japanese encephalitis to swine for preventing viremia (with specific reference to the effect of vaccination on viremia; epidemiological study on Japanese encephalitis. 35)

Masana Ogata* Yutaka Nagao† Fumihiko Jitsunari‡
Ritsuko Kikui** Naoji Kitamura††

*Okayama University,
†Okayama University,
‡Okayama University,
**Okayama University,
††Okayama University,

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Masana Ogata, Yutaka Nagao, Fumihiko Jitsunari, Ritsuko Kikui, and Naoji Kitamura

Abstract

As to trial toward the elimination of Japanese encephalitis virus in natural surroundings, pigs received inoculation of inactivated Japanese encephalitis vaccine supplemented with complete Freund's adjuvant twice at one-week interval. Effect of adjuvant supplement on the magnitude of antibody and also prevention of viremia caused by natural infection by antibody induced with vaccine were investigated. The results of this study are summarized as follows. 1. In the group of pigs inoculated with vaccine containing adjuvant, titer of hemoagglutination inhibiting and neutralizing antibodies was higher than those inoculated with vaccine alone and their high titer persisted. 2. With respect to natural infection of pigs, on August 22 when the pigs were thought to have been infected, there was observed a rise in antibody titers. And on antibody formed in those pigs inoculated with vaccine with or without adjuvant proved to be all 2-ME resistant type, whereas the antibodies produced in control group were 2-ME sensitive antibody. 3. Viremia was detected in the blood of pigs naturally infected, but it was not demonstrated pigs inoculated with vaccine supplemented with adjuvant or without adjuvant. The virus of pig blood which was inoculated into suckling mouse brain and was separated after low suckling passage mouse was supposed to be JaGAr strain from optimum hydrogen ion concentration of its hemoagglutination reaction. 4. Effect of vaccination on antibody response of pigs having maternal antibody was not recognized.

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*PMID: 4334578 [PubMed - indexed for MEDLINE]

Acta Med. Okayama **24**, 579—587 (1970)

VACCINATION WITH COMPLETE ADJUVANT-ADDED INACTIVATED VIRUS VACCINE OF JAPANESE ENCEPHALITIS TO SWINE FOR PREVENTING VIREMIA (WITH SPECIFIC REFERENCE TO THE EFFECT OF VACCINATION ON VIREMIA; EPIDEMIOLOGICAL STUDY ON JAPANESE ENCEPHALITIS 35)

Masana OGATA, Yutaka NAGAO, Fumihiko JITSUNARI, Ritsuko KIKUI* and Naoji KITAMURA*

*Department of Public Health, Okayama University Medical School and *Institute of Hygiene, Okayama Prefecture, Okayama, Japan (Director: Prof. M. Ogata)*

Received for publication, November 9, 1970

Many ecologic studies (1, 2) of Japanese encephalitis (JE) virus have recognized that swine in Japan are naturally infected in high incidence and are an important natural source of virus for *Culex tritaeniorhynchus* proved to be the vector mosquito. Therefore, it is necessary to prevent viremia in swine in order to eliminate the natural source of infection. For this purpose, we tried a method of vaccination using inactivated JE virus vaccine, but the antibody response was found very low.

Freund's adjuvant was widely used for yielding high antibody in rabbits and guinea pigs (3-6). And we developed a method of vaccination using inactivated JE virus vaccine supplemented with Freund's complete adjuvant in 1968 (7). In the previous report (7, 8), we recognized that swine inoculated JE vaccine supplemented with complete adjuvant showed higher titers of hemoagglutination-inhibiting (HI) and neutralizing (NT) antibody than those swine inoculated JE virus alone.

In the present study, we examined effects of inactivated JE vaccine supplemented with Freund's complete adjuvant on antibody response of swine having trace or lack of antibody by HI and NT tests. And we also tested whether occurrence of viremia in swine caused by natural infection can be protected by antibody induced with inoculation of vaccine or not. In addition to this, the effect of vaccination on swine having maternal HI antibody was conducted.

MATERIALS AND METHODS

Materials;

JE vaccine; JE vaccine for animal use (formalin inactivated vaccine, containing 0.01 w/v % of ethyl-mercury thiosalicylic acid, sodium salt) was used.

Adjuvant; Freund's complete adjuvant (Difco Lab.) was used.

Pigs; Pigs older than 2 months without showing any HI reaction due to maternal antibody, and pigs 1.5 month ages, showing 1 : 80 of HI antibody titer were employed. These animals were raised in Takahashi district, Okayama Prefecture, where JE virus is epidemic every year.

Methods:

Vaccination; Pigs having a trace or lacking in maternal antibody were divided into two groups. One group was inoculated with 3 ml, 3 ml and 4 ml of JE vaccine intramuscurlary in the neck, successively at one week interval. The other group was inoculated with 6 ml, 6 ml and 8 ml of mixed suspension of equal volume of JE vaccine and complete adjuvant on the same schedule. Pigs having maternal antibody were inoculated with 6 ml of mixed suspension of JE virus.

Collection of the pig serum; Blood of pigs having trace or lacking in maternal antibody was obtained from auricular vein at about one week interval after first inoculation, and on August 28, when the rise in HI titers of pigs was believed to have completed, the last lots of blood were collected. The blood of pigs having maternal antibody was taken at about one week interval. Blood was centrifuged and serum was used for determination of antibody.

Determination of antibodies in the serum;

Hemoagglutination inhibiting (HI) antibody titers in the sera of pigs were tested by the technique of the HI test following the modified method of CLARK and CASALS (9). The hemoagglutination antigen used was JaGAr strain (Takeda Co.) purified with ether after the passage through suckling mouse brain.

Neutralizing (NT) antibody titers were measured by 50 % of plaque reduction, using cultures of chick embryo fibroblast, employing the method of KODAMA, SASAKI and INOUE (10), modified method of PORTERFIELD (11). Sera were inactivated at 56°C for 30 min. before use. And the sera four dilutions from 1:2 to 1 : 8192 were mixed with an estimated 33.07 plaque forming units of the virus in the form of an infected mouse brain suspension on JaGAr 01 SME strain by succesive passage.

Equal volumes of diluted serum and virus in Hank's balanced salt solution containing 5 % antibody-free calf serum were mixed. The mixtures were incubated for 2 hours at 37°C and 0.4 ml of each one was inoculated into two drained plate cultures of PS cells. After an adsorption period of 90 min. at 37°C, the monolayers were covered with agar. The first overlay contained final concentrations of 1.0 % agar 0.5 % lactalbumin hydrolysate 0.4 % glucose, 0.05 % yeast extract and 0.225 % sodium bicarbonate in Earle's solution included one hundred unit of penicillin and 100 μg of streptmycin/ml.

After 2 days of cultivation, a second overlay containing 1.0 % agar and 1 : 10,000 neutral red in Earle's salt solution was added. Two days later, the plaque counts of both plates at each serum dilution were compared with a number of plaques in the control without antiserum. The serum dilution causing 50 % reduction in plaque count was calculated by Karber's method and neutralizing antibody titer was expressed by n of the formula 10×2^n and also this value was taken as the multiple of the serum dilution.

Detection of viremia:

Pig's blood obtained with heparin-wetted syringes on the 92 post inoculation days (August 22) was inoculated into six weanling mice. Virus was considered to be present when at least half the mice exhibited signs of infection within 14 days of inoculation.

RESULTS

Hemoagglutination inhibiting antibody response: Serum samples were obtained before inoculation and on days 21, 28, 36, 56, 70, 77, 84, 92 and 96. The results are shown in Fig. 1 and Table 1.

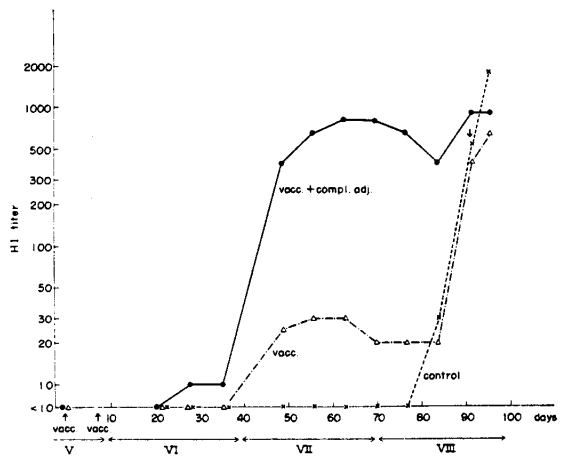

Fig. 1 Changes in HI antibody titers in the pigs inoculated with inactivated Japanese encephalitis vaccine supplemented with or without complete Freund's adjuvant, and after subsequent natural infection by Japanese encephalitis virus. Note : Arrow represents the date, when viremia in the control pigs was recognized.

The group receiving inoculation of JE vaccine with adjuvant: Pigs receiving the second inoculation on 6 days after inoculation, showed no detectable antibody. And on days 63 and 70 level of the antibody attained its maximum, showing 1 : 810 of HI titer then decreased gradually and on day 84, HI titer showed 1 : 400. Thereafter titer of antibody increased to 1 : 908 on 92 day, suggesting natural infection, which proved to be 2-ME resistant antibody.

The group receiving inoculation of JE virus alone: On 36 day after inoculation, pigs showed no detectable antibody, and on days 56 and 53, level of antibody attained its maximum, showing 1 : 30 of HI titer, then decreased gradually and titer showed 1 : 20 on 70, 77 and 84 day. Thereafter HI titer increased to 1 : 400 on 92 day and 1 : 645 on 96 day which proved to

TABLE 1. VARIATION OF LEVEL OF HI ANTIBODY, 2-ME ANTIBODY, AND NEUTRALIZING ANTIBODY IN THE CASE OF ADMINISTRATION OF INACTIVATED VACCINE WITH OR WITHOUT COMPLETE ADJUVANT TO SWINE FOLLOWED BY NATURAL INFECTION

methods	Imm. react.	date (D/M) / days after inocu. 3) / pigs / 2-ME	12/6 21	19/6 28	27/6 36
inactivated vaccine +complete adjuvant	HI 1) titers	No. 1	<10	<10	<10
		No. 2	<10	<10	<10
		No. 3	<10	20	20
		mathemat. average	<10	7 5)	7 5)
		Log-mean 4)	<10	10 6)	10 6)
	NT 2) titers	n of (10×2^n)			
		serum dilution			
inactivated vaccine inoculation	HI titers	No. 4	<10	<10	<10
		No. 5	<10	<10	<10
		No. 6	<10	<10	<10
		mathemat. average	<10	<10	<10
		Log-mean			
	NT titers	n of (10×2^n)			
		serum dilution			
non-vaccinated	HI titers	No. 7	<10	<10	<10
		No. 8	<10	<10	<10
		No. 9	<10	<10	<10
		No. 10	<10	<10	<10
		mathemat. average	<10	<10	<10
		Log-mean			
	NT titers	n of (10×2^n)			
		serum dilution			

1) HI titers; Hemoagglutination inhibiting antibody titers 2) NT titers; Neutralizing antibody titers 3) Days after first inoculation 4) Mean of Log scale 5) Calculated 10 below as zero 6) Calculated below 10 as 1

HI titers in the parentheses ( ) were mean values calculated from HI titers of No. 2 and No. 3 pigs omitting No. 1 pig for the reason of No. 1 was considered not to be naturally infected. Second inoculation was conducted 9 days after the first inoculation

be 2-ME resistant antibody.

Control group: Up to August 7, pigs showed no detectable antibody, and August 22, antibody increased markedly, showing 1 : 1600 of average HI titer by natural infection which proved to be 2-ME sensitive antibody.

TABLE 1. CONTINUED VARIATI ON OF LEVEL OFANTIBODY IN THE CASE OF ADMINISTRATION OF INACTIVATED VACCINE WITH OR WITHOUT FOLLOWED BY NATURAL INFECTION

10/7	17/7	24/7	31/7	7/8	14/8	22/8		28/8	
49	56	63	70	77	84	92		96	
						−	+	−	+
320	320	320	320	320	160	160	80	160	160
640	640	640	640	320	320	640	320	640	640
640	1280	2560	2560	2560	1280	1280	640	1280	640
530	750	1170	1170	1070	90	690 (960)	350 (480)	690 (960)	480 (640)
400	650	810	810	650	400	520 (905)	260 (450)	520 (905)	390 (640)
		6.6		6.6	7.6			7.6	
		970		970	2550			2550	
20	20	10	<10	<10	<10	2560	1280	2560	2560
20	20	20	10	10	40	640	320	640	320
40	80	160	80	80	20	40	40	160	80
26	40	63	5) 30	5) 30	5) 20	1080	547	1120	987
25	30	30	20	20	20	400	260	650	400
		6.2		3.6	3.6			8.4	
		740		120	120			3400	
<10	<10	<10	<10	<10	320	2560	40	2560	640
<10	<10	<10	<10	<10	<10	1280	160	1280	320
<10	<10	<10	<10	<10	<10	<10	<10	1280	40
<10	<10	<10	<10	<10	<10	2560	160	2560	640
<10	<10	<10	<10	<10	5) 80	5) 1600	5) 90	1920	410
					6) 30	6) 540	6) 60	1800	300
		2.4		1.4	5.0			8.4	
		53		26	320			3400	

body titer 3) Days after first inoculation 4) Mean of Log scale 5) Calculated 10 below No. 3 pigs omitting No. 1 pig for the real son of No. 1 was considered not to be naturally infected.

Summarizing these findings: It is obvious that the maximum HI antibody titer in the pigs inoculated vaccine with adjuvant as compared to that of pigs inoculated vaccine alone is 810 : 30 or 27 fold of the titer.

Neutralizing antibody response: On day 84 after the inoculation of vaccine with adjuvant the NT titer was 1 : 2550 and it was only 12 in the pigs inoculated with vaccine alone.

Viremia in pigs: JE virus was detected in the blood of two out of four control pigs naturally infected, on August 22, 1969 in the bloods. However, viremia was not demonstrated in three swine inoculated vaccine with

adjuvant and three swine inoculated vaccine alone. JE virus of blood was inoculated in suckling mouse brain and was separated by ether purification method, after suckling mouse passage, 5 times. Thereafter, hemoagglutination reaction at various range of hydrogen ion concentration was tested. Optimum hydrogen ion concentration of separated virus was pH 7.8 (Table 2). As optimum hydrogen ion concentration of hemoagglutination reaction of Nakayama strain was reported as pH 7.8 and that of JaGAr was as pH 7.4, the isolated virus was supposed to be JaGAr strain.

TABLE 2 HEMOAGGLUTINATION REACTION OF JAPANESE ENCEPHALITIS VIRUS ISOLATED FROM BLOOD OF SWINE IN TAKAMATSU DISTRICT, AT VARIOUS HYDROGEN ION CONCENTRATION

pH \ Dil.	100	200	400	800	1.600	3200	6400	12800	saline
6.2	+	+	+	+	−	−	−	−	−
6.4	+	+	+	+	−	−	−	−	−
6.6	+	+	+	+	±	−	−	−	−
6.8	+	+	+	+	+	−	−	−	−
7.0	+	+	+	+	±	−	−	−	−

Dil : Dilution of isolated virus
+ : Positive agglutination
± : Trace agglutination

Effect of vaccination on antibody production of pigs having maternal antibody in the sera: Pigs which recognized maternal antibody of 1 : 80 of HI antibody titer in the sera were inoculated with inactivated vaccine supplemented adjuvant and HI antibody response was determined. Decrease in the level of HI antibody titer of pigs with inoculation after time lapses was similar to those without inoculation, and 2-ME sensitive antibody

TABLE 3. VARIATION OF LEVEL OF HI ANTIBODY, AFTER INOCULATION OF INACTIVATED JE VACCINE WITH COMPLETE ADJUVANT TO SWINE BORN IN 17TH OF APRIL HAVING MATERNAL ANTIBODY

vacc. method	data (D/M)	1/6	8/6	26/6		14/7
	day after inoc.	0	7	20		43
	pigs \ 2-ME	−	−	−	+	−
inactivated vaccine + complete adjuvant inoculation	No. 1	80	40	20	10	10
	No. 2	80	40	20	20	10
	No. 3	80	40	20	20	10
	No. 4	80	40	20	10	10
	mathemat. average	80	40	20	15	10
non-vaccinated	No. 1	80	40	20	10	10
	No. 2	80	40	20	10	10
	average	80	40	20	10	10

was not recognized in the serum on 20 days after inoculation as shown in Table 3. Therefore, HI antibody response was not recognized in pigs having maternal antibody.

DISCUSSION

Up to date, two inoculation methods have been considered for vaccination of JE virus in pigs, inoculation with live attenuated vaccine and that with inactivated vaccine.

KODAMA, SASAKI and INOUE (9) inoculated pigs with an attenuated mutant (m) strain of JE virus and found that strain produced no detectable viremia and was able to evoke both hemoagglutinin-inhibiting (HI) and neutralizing (NI) antibodies in colostrum deprived pigs after a single subcutaneous injection.

There arises a problem of back mutation in the inoculation of live vaccine to pigs and there is also a disadvantage of less elevation in the antibody level in the case of inoculation with inactivated JE vaccine. For the purpose to overcome these disadvantages, we have devised a method of inoculation with inactivated JE vaccine supplemented with complete adjuvant.

The immunization method of protein antigen supplemented with Freund's complete adjuvant was characterized by its feature of maintaining high antibody titers in the rabbits, chicks and guinea pigs. (3-5). An attempt has been made for application of swine with inactivated JE vaccine.

In our study on the effects of inactivated JE vaccine supplemented with complete adjuvant in swine, it has been demonstrated that the vaccination by this method yields higher HI and neutralizing antibody titers than that with vaccine alone, and the antibody titer can be maintained at high level. As the pigs inoculated with such a vaccine have high antigen titers, it is thought that the onset of viremia can be prevented in pigs when a considerable lapse of time after the vaccination.

As to the relation between the dose of vaccine and magnitude of antibody response, maximum level of HI antibody of pigs received inoculation of vaccine with adjuvant twice, 2 ml in each showed average titer of 1 : 200 as described previously (7), and those inoculated three times, 6 ml, 6 ml and 8 ml respectively was 1 : 810 as shown in this experiment.

In the case of pigs inoculated vaccine alone, the maximum level (7) of HI titers in the sera of two pigs inoculated with 1 ml vaccine twice was 1 : 10 and below 1 : 10 respectively, showing possibility of inducing viremia

as described in later. Pigs inoculated with vaccine alone showed different HI titer each other in this experiment, No. 4 and No. 5 swines showed HI titer of below 10 and 10 respectively and No. 6 showed HI titer of 80 on July 31 and August 7.

SHERER, *et al.* (2) examined viremia in pigs naturally infected, while in mosquito traps, and three pigs demonstrated viremia, one pig showed an HI titer of 1 : 10 in the serum and the other two showed HI titers of below 10. Therefore, possibility of viremia in pigs inoculated vaccine alone is considerable, though we could not detect viremia in the blood.

In our experiment effect of vaccination on antibody response of pigs having maternal antibody was not recognized. As to the reason, the incomplete antibody production of pigs in young age or neutralization of vaccine at injected place and blood by maternal antibody can be considered. As colostrum deprived pig could be produced HI and NT antibody as described by KODAMA, SASAKI and INOUE (9), the neutralizing of vaccine by maternal antibody was taken into consideration.

CONCLUSION

As to trial toward the elimination of Japanese encephalitis virus in natural surroundings, pigs received inoculation of inactivated Japanese encephalitis vaccine supplemented with complete Freund's adjuvant twice at one-week interval. Effect of adjuvant supplement on the magnitude of antibody and also prevention of viremia caused by natural infection by antibody induced with vaccine were investigated. The results of this study are summarized as follows.

1. In the group of pigs inoculated with vaccine containing adjuvant, titer of hemoagglutination inhibiting and neutralizing antibodies was higher than those inoculated with vaccine alone and their high titer persisted.

2. With respect to natural infection of pigs, on August 22 when the pigs were thought to have been infected, there was observed a rise in antibody titers. And on antibody formed in those pigs inoculated with vaccine with or without adjuvant proved to be all 2-ME resistant type, whereas the antibodies produced in control group were 2-ME sensitive antibody.

3. Viremia was detected in the blood of pigs naturally infected, but it was not demonstrated pigs inoculated with vaccine supplemented with adjuvant or without adjuvant. The virus of pig blood which was inoculated into suckling mouse brain and was separated after low suckling passage mouse was supposed to be JaGAr strain from optimum hydrogen ion concentration of its hemoagglutination reaction.

4. Effect of vaccination on antibody response of pigs having maternal antibody was not recognized.

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