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Vaccination with complete adjuvant-added inactivated virus vaccine of Japanese encephalitis to swine, rabbits and chicks for preventing viremia (epidemiological study on Japanese encephalitis 25)

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Abstract

As a step towards the elimination of Japanese encephalitis virus in natural surroundings, we inoculated pigs, rabbits and chicks with inactivated Japanese encephalitis vaccine supplemented with complete or incomplete Freund's adjuvant twice at one-week interval. Subsequently, we compared HI antibody titers of the groups inoculated with vaccine containing complete Freund's adjuvant (pigs, rabbits, chicks), of the group inoculated with vaccine containing incomplete adjuvant (rabbits), and of the groups inoculated with vaccine containing no adjuvant (pigs, rabbits, chicks), and also observations on changes in the antibody titers due to natural infection. In a certain portion of these animals neutralizing antibody titers were also determined. The results of this study are briefly summarized as follows. 1. In the groups of pigs and rabbits inoculated with vaccine containing complete Freund's adjuvant, titers of HI antibody and neutralizing antibody were higher than those inoculated with vaccine containing no adjuvant and their high titers persisted. Further, in the group of chicks inoculated with inactivated Japanese encephalitis vaccine containing complete Freund's adjuvant, HI antibody titers were higher and persistent as compared with the antibody titers in the chicks inoculated with inactivated Japanese encephalitis vaccine alone. 2. In the rabbits inoculated with inactivated Japanese encephalitis vaccine containing incomplete adjuvant, HI antibody titers were lower than in those receiving the vaccine with complete adjuvant, but it has been demonstrated clearly that vaccination of inactivated Japanese encephalitis vaccine supplemented with incomplete adjuvant brings about less sideeffects. Hence such a method of vaccination can be applied as the vaccination with least side-effects. 3. With respect to natural infection of swine, on August 27 when the pigs were thought to have been infected, there was observed a rise in antibody titers. And on being infected with Japanese encephalitis, the antibodies formed in those pigs inoculated with inactivated Japanese encephalitis vaccine with or without complete adjuvant proved to be all 2-ME resistant type, whereas the antibodies produced in the control groups not receiving such a vaccination were 2-ME sensitive antibody.

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**VACCINATION WITH COMPLETE ADJUVANT-ADDED
INACTIVATED VIRUS VACCINE OF JAPANESE ENCE-
PHALITIS TO SWINE, RABBITS AND CHICKS FOR
PREVENTING VIREMIA (EPIDEMIOLOGICAL STUDY
ON JAPANESE ENCEPHALITIS 25)**

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It is said that Japanese encephalitis (JE) virus propagates in vectors such as swine (1) and herons (2—3), and when mosquitoes suck the blood of these vectors while in the state of viremia, the mosquitoes in turn transmit JE virus to man causing Japanese encephalitis in some of them. As reported by BUESCHRE and SCHERER *et al.* (4), there is also a possibility of source of JE infection other than swine and herons. We have demonstrated that when chicks are naturally infected with JE virus in summer, there can be observed high titers of hemagglutination inhibiting (HI) antibody and mercaptoethanol (2-ME) sensitive antibody (5), indicating that chicks can also be the source of JE infection. We have pointed out previously that cattle and dogs (6) can be carriers of the virus.

Therefore, it is necessary to prevent viremia in these possible vectors in order to eliminate the source of infection. To accomplish this, we must vaccinate these vectors with JE vaccine to prevent viremia. For this purpose we have developed a method of vaccination using inactivated JE vaccine supplemented with FREUND'S complete adjuvant by which rabbits can be immunized (7, a, b, 8). This method is characterized by its feature (7 c) of maintaining high antibody titers in the animals inoculated. In our tests conducted with swine inoculated by this method during the winter season when mosquitoes are dormant, we find that pigs inoculated JE vaccine supplemented with complete adjuvant show higher titers than those pigs inoculated JE vaccine alone.

In the present study we examined effects of inactivated JE vaccine supplemented with FREUND'S complete adjuvant by subsequent HI tests on swine, rabbits and chicks and neutralization tests (NT test). With pigs we determined whether there occurred secondary response, when infected naturally, by means of 2-ME tests. With rabbits, we also studied effects

of the vaccine supplemented with FREUND's incomplete adjuvant on the rise in titers. The present communication deals with the results of such vaccination.

MATERIALS AND METHODS

Materials:

Japanese encephalitis (JE) vaccine; Japanese encephalitis vaccine for animal use inducing high HI and NT antibody titers (formalin inactivated vaccine, containing 0.01 w/v % of ethyl-mercury thiosalicylic acid, sodium salt, inactivated vaccine in short) was used.

Adjuvant; FREUND's complete and incomplete adjuvants (Difco Lab.) were used.

Animals; Pigs older than 2 months without showing any HI reaction due to maternal immunity were employed. These animals were selected from Takahashi District, Okayama-ken, where Japanese encephalitis virus is endemic every year. Rabbits used were older than 6 months, all showing no response to the HI tests due to maternal immunity. These animals were raised at the Okayama University Medical School. Chicks were older than 2 months, showing no maternal immunity and also raised at the same university.

Methods:

Vaccination and time of collecting blood; With pigs, the first vaccination was given on July 5 after collecting blood. Then collecting blood again on July 11 the second vaccination was performed. Thereafter blood was collected at one-week interval, and on August 27 (53 days after the first vaccination) when the rise in HI titers of pigs was believed to have completed, the last lots of blood were collected.

As for rabbits, after collecting blood on February 12 the first vaccination was done. Then collecting blood on February 19, the second vaccination was conducted. Thereafter blood was collected at one-week interval, and the final blood was collected on September 9, when the rise in HI titers in rabbits was thought to have completed by natural infection. With chicks after drawing blood on June 21, the first vaccine was given, and collecting blood again on June 28, the second vaccination was performed.

Vaccination methods; Pigs were divided into two groups, one of which received 1 ml of inactivated Japanese encephalitis vaccine without adjuvant intramuscularly to the neck, and the other group, suspension (1 ml) of the inactivated Japanese encephalitis vaccine supplemented with 1 ml complete FREUND's adjuvant also intramuscularly into the neck. For rabbits the vaccination was done intramuscularly on the back and for chicks likewise intramuscularly to the breast.

Methods of collecting blood; From pigs, blood was collected from auricular vein, from rabbits by heart puncture, and from chicks from wing vein.

Immunological reaction; The titration of Japanese encephalitis HI antibody and 2ME sensitive antibody of Japanese encephalitis was done by the method described by CLARK and CASALS (9).

The estimation of JE neutralizing antibody titers was done by the CE plaque method (10). The oncogenic virus used was JaGar 01 SME strain maintained by successive passage. To each serum of gradient dilution, the antigenic solution was added and with each Petri dish the number of plaque was counted. The neutralizing antibody titers were calculated by the multiple of dilution of the serum that showed 50% decrease in plaque number, and the quantity of the virus used was 33.07 P. F. units. Namely, the neutralizing antibody titers were expressed by n of the formula 10×2^n , and also this value was taken as the multiple of the serum dilution. In Table 1, the figures enclosed in parentheses represent the values of n , and the figures outside the diluted antibody titers.

RESULTS

Inoculation of inactivated JE vaccine with complete FREUND's adjuvant to pigs :

Changes in HI titers : As shown in Fig. 1, the group receiving the second inoculation of inactivated Japanese encephalitis vaccine with adjuvant on day 6 after the first vaccination showed less than 10 of HI titers, and on day 19 the mathematical average of the titers was the maximum of 240. This value gradually decreased and on day 38 it was down to 53. On day 53 (August 27) the titer rose to the level which suggested of natural infection, giving the titer of 960, which proved to be 2-ME resistant antibody. In contrast, in the group inoculated with vaccine containing no adjuvant, No. 4

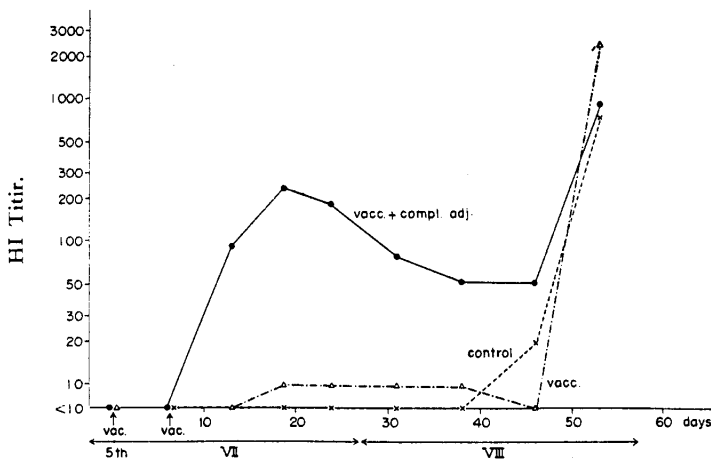


Fig. 1 Changes in HI antibody titers in the pigs inoculated with inactivated Japanese encephalitis vaccine supplemented with or without complete Freund's adjuvant, and after subsequent natural infection by Japanese encephalitis virus.

Note, HI antibody titer of No. 4 pig was taken as a representative one of the vaccinated group. For convenience the antibody titer on day 46 was made zero.

animal (Table 1) showed HI titers of less than 10 on day 13 and 10 on day 19. This titer was maintained up to day 38, and on day 53 when there was a rise in the titers suggesting natural infection, the value reached as high as 2,560. This antibody proved to be 2-ME resistant antibody. No. 5 animal showed antibody titer of less than 10 up to day 46 after the first inoculation, but on day 53 when the titer rose to suggest of natural infection, the titer reached up to 2,560. Its HI antibody titer then was decreased to 1,280 by 2-ME test, and the formation of 2-ME resistant antibody was recognized though not so high as No. 4 animal. In the group of pigs not vaccinated as the control, up to August 12 (38 days after the vaccination of the comparable test group) HI titer was less than 10, and on day 53 at the time believed to show the rise in antibody due to natural infection the average antibody titer was 800, and such antibodies in every case proved to be all 2-ME sensitive type. Summarizing these findings, it is obvious that the maximum HI antibody titer in the pigs inoculated with inactivated Japanese encephalitis vaccine supplemented with complete FREUND's adjuvant as compared to that of No. 4 pig inoculated with the vaccine without the adjuvant is 240 : 10 or 24-fold of the latter, and the same titer on day 38 in 53 : 10. In other words, the inoculation of the vaccine supplemented with adjuvant seems to be a very effective method of yielding high HI antibody titer.

Changes in the neutralizing antibody titer : Changes in the neutralizing titer are clearly demonstrated by the plaque decreasing test. In other words, on day 19 after the inoculation of the Japanese encephalitis vaccine supplemented with adjuvant, the neutralizing titer is 730 as against only 12 in the pig inoculated with the Japanese encephalitis vaccine without adjuvant.

Inoculation of inactivated Japanese encephalitis vaccine with adjuvant to rabbits :

Alterations in the HI titer : As illustrated in Table 2 and Fig. 2, titer reaches up to 320 by 35 days after the first inoculation of vaccine supplemented with complete adjuvant. Thereafter it gradually decreased, reaching 80 on day 107 and down to 40 by day 193. And October 9 corresponding to 240 days after the first inoculation when there is a rise in the titer believed to be due to natural infection, HI antibody titer has increased to 80. In the group receiving inactivated Japanese encephalitis vaccine supplemented with incomplete FREUND's adjuvant, the average of HI antibody titers during the period between 21 and 35 days after the first inoculation reaches its maximum of 120. Gradually decreasing thereafter, it is down to 40 on day 70, and one rabbit showed the value of

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. reac.	date (D/M)	5/7		11/7		18/7		24/7		29/7		5/8		12/8		20/8		27/8	
		days after inocul. ⁵⁾	0		6*		13		19		24		31		38		46		53	
		pigs	2-ME		-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
inactivated vaccine + complete adjuvant inoculation	HI 1) titers	No. 1	<10	<10	40	40	80	40	80	40	80	40	80	40	80	40	80	40	640	320
		No. 2	<10	<10	160	80	320	160	320	160	320	160	80	40	40	20	40	20	1280	640
		No. 3	<10	<10	80	20	320	80	160	80	160	80	80	40	40	20	40	20	[40]	[20]
		mathemat. average ³⁾	<10	<10	93	47	240	93	187	93	80	40	53	27	53	27	960	480		
		Log-mean ⁴⁾	<10	<10	80	40	200	80	160	80	80	40	50	25	50	25	910	450		
	NT 2) titers	Log-mean			(5.1)**	340	(6.2)**	730												
inactivated vaccine inoculation	HI titers	No. 4	<10	<10	<10		10		10		10		10		10		<10		2560	2560
		No. 5	<10	<10	<10		<10***		<10***		<10***		<10***		<10***		<10		2560	1280
		mathemat. average	<10	<10	<10		5***		5***		5***		5***		5***		<10		2560	1920
		Log-mean																	2560	1810
	NT titers	Log-mean					(0.3)	12												
non -vaccinated	HI titers	No. 6	<10	<10	<10		<10		<10		<10		<10		<10		<10		320	20
		No. 7	<10	<10	<10		<10		<10		<10		<10		<10		40***		1280	40
		mathemat. average	<10	<10	<10		<10		<10		<10		<10		<10		20***		800	30
		Log-mean																	640	28
	NT titers	Log-mean																(>8)	2560	

1) HI titers; Hemagglutination inhibiting antibody titers 2) NT titers; Neutralizing antibody titers 3) Mean 4) Mean of Log 5) Days after first inoculation
 *) Second inoculation **) n values of 10×2ⁿ in the parentheses ***) Calculated 10 below as zero
 HI titer in the parentheses [] were discarded for calculation of mean, as not having been naturally infection.

Table 2. Variation of level of HI antibody, and neutralizing antibody in the case of administration of inactivated vaccine with or without complete adjuvant or incomplete adjuvant to rabbits, followed by natural infection

methods	imm. reac.	date (D/M) days after inocul ⁵⁾	19/2	26/2	4/3	11/3	18/3	25/3	2/4	8/4	15/4	22/4	30/4	9/5	29/5	20/6	17/7	23/8	9/10		
			7*	14	21	28	35	42	50	56	63	70	78	87	107	129	156	193	240		
vaccine 1 ml + complete adjuvant 1 ml	HI ¹⁾ titers	No. 1	<10	10	20	80	320	160	160	death	—	—	—	—	—	—	—	—	—	—	
		No. 2	20	80	160	320	320	160	160	160	160	80	80	80	80	80	80	40	40	80	
		No. 3	<10	40	80	160	320	160	160	160	160	160	160	160	160	80	80	80	40	40	80
		mathemat. aver. ³⁾	7	43	87	187	320	160	160	160	160	160	120	120	120	80	80	60	40	80	
	Log-mean ⁴⁾		32	64	160	320	160	160	160	160	160	113	113	113	80	80	57	40	80		
NT ²⁾ titers	Log-mean			240** (4.7)		450 (5.5)	1280 (7.0)								230 (4.5)						
vaccine 1 ml + incomplete adjuvant 1 ml	HI titers	No. 4	<10	20	80	80	80	80	80	80	40	40	death	—	—	—	—	—	—		
		No. 5	<10	80	160	160	160	80	80	80	80	40	40	40	40	20	10	10	40		
	NT titers	Log-mean		40	113	113	113	80	80	80	57	40									
vaccine ml	HI titers	No. 6	10	40	40	20	20	10	10	10	10	10	10	<10	<10	<10	<10	<10	<10	10	
		No. 7	20	40	40	40	20	20	20	20	20	20	20	20	<10	<10	<10	<10	<10	20	
		mathemat. aver.	15	40	40	30	20	15	15	15	15	15	15	15	10	<10	<10	<10	<10	15	
	NT titers	Log-mean			60 (2.6)																

* Second inoculation

** Titers inside the parentheses are numbers of dilution showing fifty decrease of plaque, and titers outside the parentheses are those calculated with the number of dilution.

Dose of virus used; \bar{x} =57.29.

Symbols on the upper right are the same as in Table 1.

Vaccination for Preventing Viremia

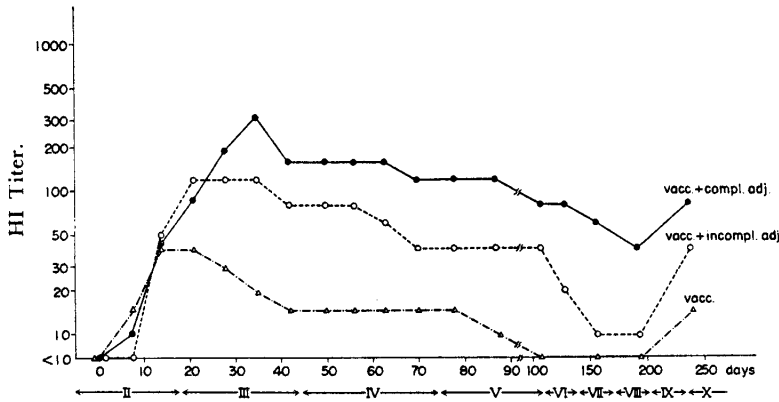


Fig. 2 Changes in HI antibody titers in the rabbits receiving inactivated Japanese encephalitis vaccine with or without complete or incomplete Freund's adjuvant and after natural infection.

10 on day 156. On October 9, corresponding to 240 days, there was a rise in the antibody titer believed to be due to natural infection revealing as high value as 40. In contrast, with the group of rabbits receiving the vaccine without adjuvant the average of HI antibody titers reached its maximum of 40 between 14 and 21 days, and decreasing gradually thereafter, it dropped to 15 between the periods of 42 days and 78 days. Between days 107 and 193 it was under 10. On October 9 (240 days) there was a rise in the antibody titer suggesting of natural infection, showing HI antibody titer of 15.

Changes in the neutralizing antibody titer : In the group given inactivated Japanese encephalitis vaccine with complete FREUND'S adjuvant, maximum titers of neutralizing antibody, as revealed by the plaque-decreasing test, were 240 on day 21 after the first inoculation; 450 on day 35, 1280 on day 42 and 230 on day 107. In the group receiving the inactivated Japanese encephalitis vaccine without adjuvant the neutralizing antibody titer was 60 on day 21 after the first inoculation, as compared with showing only less than 1/4 the value 240 in the group receiving the vaccine with complete adjuvant. In other words, even the neutralizing antibody titer, which is believed to represent the direct resistance potency of serum antibody to infection, is clearly high in the group receiving the vaccine supplemented with complete adjuvant than in the group inoculated with the vaccine without adjuvant.

Comparison among three vaccination methods : These results indicate that for increasing antibodies to Japanese encephalitis, the inactivated Japanese

encephalitis vaccine with complete FREUND'S adjuvant, the inactivated Japanese encephalitis vaccine with incomplete FREUND'S adjuvant, and the inactivated Japanese encephalitis vaccine without adjuvant are effective in the order mentioned, and that the vaccine supplemented with incomplete FREUND'S adjuvant which has less side-effect gives higher HI antibody titer than normal, though not quite so effectively as the vaccine with complete adjuvant and it can maintain high level of the antibody.

Table 3 Variation of level of HI antibody in the case of administration of inactivated vaccine with or without complete adjuvant to chicks

methods	Date (D/M) days after inocul. chicks	HI antibody titers				
		21/6	28/6	12/7	23/8	31/10
		0	7*	21	63	132
vaccine 1 ml + complete adjuvant 1 ml	No. 1	< 10	< 10	160	160	40
	No. 2	< 10	20	160	320	80
	No. 3	< 10	< 10	160	160	80
	No. 4	< 10	< 10	160	160	80
	mathemat. average	< 10	5	160	200	70
	Log-mean	< 10	5	160	190	67
vaccine 1 ml	No. 5	< 10	20	80	40	20
	No. 6	< 10	< 10	< 10	< 10	< 10
	No. 7	< 10	< 10	40	80	80
	No. 8	< 10	40	80	20	20
	mathemat. average	< 10	15	50	35	30

* Second inoculation

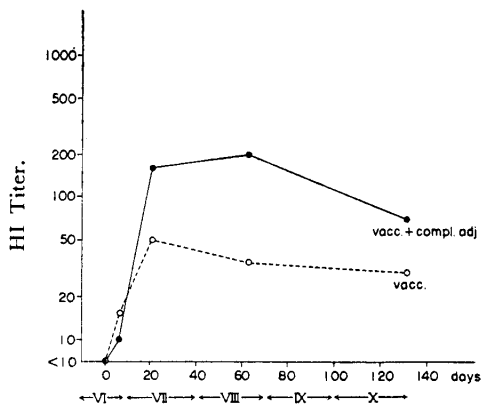


Fig. 3 Changes in HI antibody titers in the chicks receiving inactivated Japanese encephalitis vaccine with or without complete adjuvant.

Inoculation of inactivated Japanese encephalitis vaccine supplemented with complete Freund's adjuvant to chicks: Changes in the HI antibody titer and the neutralizing antibody titer after the inoculation of Japanese encephalitis vaccine to chicks are shown in Table 3 and Fig. 3. In the group of chicks inoculated with inactivated Japanese encephalitis vaccine with complete FREUND'S adjuvant, HI antibody titer was 160 in average 21 days after the

second inoculation whereas it was under 10 prior to the first inoculation, and it rose to 200 on day 63 and fell to 70 on day 132. On the other hand, in the group receiving the vaccine without adjuvant the average titer of HI antibody 21 days after the first inoculation was the maximum of 50 and 35 on day 63, then 30 on day 132. In other words the maximum titer of this group was about 1/4 the HI antibody titer of the group receiving the inoculation of inactivated Japanese encephalitis vaccine with complete FREUND'S adjuvant. These results indicate that the inoculation of inactivated Japanese encephalitis vaccine with complete adjuvant has advantages of yielding high HI antibody titer and maintaining it at high level. In addition this method of inoculation shows little individual differences.

DISCUSSION

Effects of inoculation of inactivated Japanese encephalitis vaccine supplemented with complete and incomplete Freund's adjuvant.

There arises a problem of back mutation in the inoculation of live vaccine to swine and there is also a disadvantage of less elevation in the antibody titer in the case of inoculation with inactivated Japanese encephalitis vaccine. For the purpose to overcome these disadvantages we have devised a method of inoculation with inactivated Japanese encephalitis vaccine supplemented with complete Freund's adjuvant. In our study on the effects of inactivated Japanese encephalitis vaccine with complete Freund's adjuvant in swine, rabbits and chicks, it has been demonstrated that the vaccination by this method yields higher HI antibody titers than that with the vaccine containing no complete Freund's adjuvant and further by this method the antigen titer can be maintained at high level. As the animals inoculated with such a vaccine have high antigen titers, it is thought that the onset of viremia can be prevented in these animals when a considerable lapse of time after the vaccination. Since incomplete Freund's adjuvant contains no mycobacteria, there is clearly less side-effect as compared with the vaccine containing complete FREUND'S adjuvant. And this vaccination acts or produces higher titers of HI and neutralizing antibodies than the vaccination of vaccine alone, though its antibody-raising capacity is less than in the case with complete adjuvant.

Neutralizing antibody and HI antibody titers : The inoculation of inactivated Japanese encephalitis vaccine supplemented with complete Freund's adjuvant to swine produces higher neutralizing antibody titer than the inoculation of the Japanese encephalitis vaccine alone, and this result

parallels with that of HI antibody. The same is true in the case of rabbits. As shown in Tables 1, 2, the titer of neutralizing antibody is higher than that of HI antibody, and this titer parallels with the antibody titer as determined by HI test. This result coincides with the results of KATSURADA's report (14) on the relationship between two antibodies as observed by the rise in antibody titers among Hokkaido citizens inoculated with Japanese encephalitis vaccine.

Properties of the antibody formed by natural infection after inoculation with Japanese encephalitis vaccine : In the case of swine naturally infected, there can be seen the formation of 2-ME sensitive antibody by the HI tests, whereas in the swine inoculated with the Japanese encephalitis vaccine and later infected there is observed the formation of 2-ME resistant antibody. This fact coincides well with the finding (15) that there is formed 2-ME sensitive antibody in patients not receiving vaccination for Japanese encephalitis while there is formed 2-ME resistant antibody in the patient receiving Japanese encephalitis vaccination.

CONCLUSION

As a step towards the elimination of Japanese encephalitis virus in natural surroundings, we inoculated pigs, rabbits and chicks with inactivated Japanese encephalitis vaccine supplemented with complete or incomplete Freund's adjuvant twice at one-week interval. Subsequently, we compared HI antibody titers of the groups inoculated with vaccine containing complete Freund's adjuvant (pigs, rabbits, chicks), of the group inoculated with vaccine containing incomplete adjuvant (rabbits), and of the groups inoculated with vaccine containing no adjuvant (pigs, rabbits, chicks), and also observations on changes in the antibody titers due to natural infection. In a certain portion of these animals neutralizing antibody titers were also determined. The results of this study are briefly summarized as follows.

1. In the groups of pigs and rabbits inoculated with vaccine containing complete Freund's adjuvant, titers of HI antibody and neutralizing antibody were higher than those inoculated with vaccine containing no adjuvant and their high titers persisted. Further, in the group of chicks inoculated with inactivated Japanese encephalitis vaccine containing complete Freund's adjuvant, HI antibody titers were higher and persistent as compared with the antibody titers in the chicks inoculated with inactivated Japanese encephalitis vaccine alone.
2. In the rabbits inoculated with inactivated Japanese encephalitis

vaccine containing incomplete adjuvant, HI antibody titers were lower than in those receiving the vaccine with complete adjuvant, but it has been demonstrated clearly that vaccination of inactivated Japanese encephalitis vaccine supplemented with incomplete adjuvant brings about less side-effects. Hence such a method of vaccination can be applied as the vaccination with least side-effects.

3. With respect to natural infection of swine, on August 27 when the pigs were thought to have been infected, there was observed a rise in antibody titers. And on being infected with Japanese encephalitis, the antibodies formed in those pigs inoculated with inactivated Japanese encephalitis vaccine with or without complete adjuvant proved to be all 2-ME resistant type, whereas the antibodies produced in the control groups not receiving such a vaccination were 2-ME sensitive antibody.

REFERENCE

1. WILLIAM, F. SCHERER, *et al.*: Immunologic Studies of Japanese Encephalitis Virus in Japan, V. Maternal Antibodies, Antibodies, Antibody Responses and Viremia following Infection of Swine. *J. Immuno.* **83**, 620, 1959
2. EDWARD, L., BUESCHER, WILLIAM, F. and SCHERER, *et al.*: Immunologic Studies of Japanese Encephalitis Virus in Japan, IV, Maternal Antibody in Birds. *J. Immuno.* **83**, 614, 1959
3. OGATA, M. and NAGAO, Y. *et al.*: Studies on the 2-Mercaptoethanol Sensitive Antibody to Japanese Encephalitis in the Serum of the Wild Birds, Chicken and Patients of Japanese Encephalitis. *Physico-Chemical Biology* **12** (3), 187, 1967 (in Japanese)
4. EDWARD, L., BUESCHER, WILLIAM, F. SCHERER, *et al.*: Immunologic Studies of Japanese Encephalitis Virus in Japan, III, Infection and Antibody Responses of Birds. *J. Immuno.* **83**, 605, 1959
5. OGATA, M., NAGAO, Y., *et al.*: Maternal Hemagglutination Inhibition Antibodies to Japanese Encephalitis Virus in Domestic Chickens. *Physico-Chemical Biology* **13** (2), 159, 1968 (in Japanese)
6. OGATA, M., NAGAO, Y., *et al.*: Seasonal Level of Antibody and its 2-ME sensitive Antibody in the Sera of Swine, Canine, Bovine and Human Inhabitants. *Japanese Journal of Public Health* **15** (6), 539, 1968 (in Japanese)
7. a) FREUND, J.: *Ann. Rev. Microbiol.* **1**, 291, 1947. b) FREUND *J. Clin. Path.* **21**, 645, 1951. c) FREUND, J.: The Effectiveness of Tuberculo-glycolipid as an Adjuvant in Eliciting Allergic Encephalomyelitis and Aspermatogenesis. *J. Immuno.* **82** (6), 1959
8. EDWARD, E., FISCHER, ELVIN, A. KABAT, *et al.*: The Role of Tubercle Bacilli in Adjuvant Emulsions on Antibody Production to Egg Albumin. *J. Immuno.* **69** (6), 611, 1952
9. CLARKE, D. H. and CASALS, J.: Techniques for Hemagglutination and Hemagglutination Inhibition with Arthropode-borne Viruses. *Am. J. Trop. Med. & Hyg.* **7**, 561, 1958
10. PORTERFIELD, J. S.: *Immunological Methods*. p.341, Edited by J. E. Ackroyd, Blackwell Scientific Publications, Oxford, 1964