Some Problems of Bacterial and Viral Infection in Mice and the Ecology of Japanese Encephalitis Virus in Japan

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Abstract: The administration of arginine to mice before infection restrained the worse changing of infection with Salmonella paratyphi B. On the other hand, the mutant of Salmonella paratyphi B which require the arginine decreased the pathogenic potentiality to mice than the original strain. However, the pathogenicity of the mutant in mice in vivo increased than the original strain. The effect of arginine on the interaction between mice and Salmonella paratyphi B was discussed. Secondly, the hemolysis of Escherichia coli was studied and extracted from the culture fluid by the isoelectric method. Thirdly, the abnormalities and absorptions of fetuses in the uterus of infected mother mice with rubella virus and the high mortality of newborns within two weeks after birth were observed. The pregnant mice were shown to be the highly susceptible host for the studing experimental rubella infection and it was of considerable value in the study of the transplacental transmission. Fourthly, the morphology of Japanese encephalitis virus was discussed with reference to the detection of the membranous structure. Fifthly, the ecology of Japanese encephalitis virus, particularly, the problems of over-wintering of the virus was described.

The author will retire from the Institute for Tropical Medicine, Nagasaki University on April, 1st 1983. Consequently, among the reports published since 1958 by the author, the several interest problems concerning the bacterial and viral infection in mice, some biological properties of Escherichia coli, and the morphology and the ecology of Japanese encephalitis virus will be introduced in the following summarized descriptions.

Ⅰ. *The biochemical condition in the infection of mice with Salmonella paratyphi B* (Nagasaki Medical Journal, 33: 653-661, 764-767, 1958).

The relationship between the host cell and the pathogenic bacteria is complicated by the interaction of many enviromental factors, especially, the metabolic substances of

Received for publication, November 5, 1982.
Contribution No. 1251 from the Institute for Tropical Medicine, Nagasaki University.

both living cells and bacteriae in the course of infection. The experiment was carried out by the combination of mice and Salmonella paratyphi B. From the biochemical point of view, the effect of amino acids on the infection of mice was studied. The mice were administrated amino acids before infection and the mutant of Salmonella paratyphi B which reqiure the arginine was applied. At the same time, the mice which were administrated without amino acids and the original strain of Salmonella paratyphi B were used comparatively.

Eighteen amino acids and thirteen related substances in the serum and extracts of liver and spleen from a normal mouse were identified by the paper-chromatography as shown in Figure 1. Among these substances, phenylalanine, arginine, methionine, leucine, valine and X_9 substance in serum and extracts vanished by the infection of four strains of Salmonella. The administration of phenylalanine, arginine and methionine by the oral or intraperitoneal routes before infection restrained the worse progress of infection with Salmonella paratyphi B. The antibody production of these mice was higher in titer of the agglutination reaction than those of mice which were not admin-

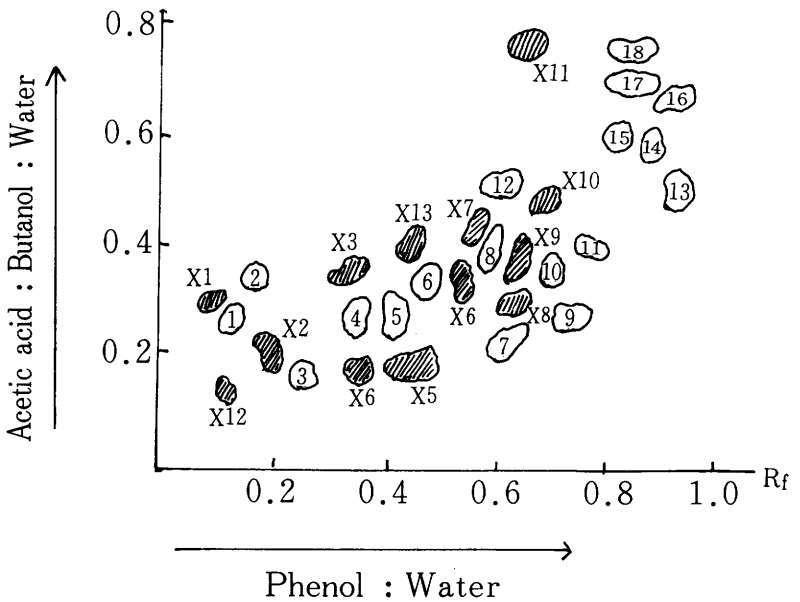

Figure 1. Paper chromatography of serum and extracts from liver and spleen of normal mouse

1. asparagic acid	2. glutamic acid	3. cystine
4. serine	5. glycine	6. threonine
7. lysine	8. alanine	9. arginine
10. oxyproline	11. histidine	12. tyrosine
13. proline	14. methionine	15. valine
16. tryptophan	17. phenylalanine	18. leucine

Xl to X13 showed the amino acid related substances.

istrated amino acids mentioned above or administrated other amino acids before infection. It was noted that any objective symptoms were not observed in these mice, though a large amount of bacteria could be detected in various organs, such as spleen, liver, mesenterial lymphnodes and sometimes in blood (bacteriaemia). When the mice restrained the infection were administrated the hypertonic glucose solution, the abrupt weak and death were introduced. The mutant of Salmonella paratyphi B which require the arginine showed the lower potentiality in the pathogenicity for mice than the parent strain. However, the mutant strain increased the pathogenicity for mice which were administrated the arginine before infection and the bacteria was disseminated in various organs, especially in blood. It was pointed out the mechanism of these phenomenon induced by the arginine was still obscure.

Table 1. Alteration of amino acids and fatty acids in serum and extracts from liver and spleen of mice challenged with S. paratyphi B, S. cholerae suis and S. enteritidis.

sample	strain	amino acid			new product	fatty acid vanished
		vanished	decreased	increased		
serum	PB8006		X_9, leucine, phenylalnine			
	PB6617					
	S. chol. suis	X_9, leucine, phenylalanine	lysine			
	S. enter.	X_9, leucine, phenylalanine				
liver	PB8006	X_9,X_8,X_7,X_6, tyrosine	arginine, valine, phenylalanine, methionine		X_{12}	gluconic acid
	PB6617	X_9,X_1, cystine, proline	X_{10},X_6, tyrosine, meth onine			
	S. chol. suis	X_9,X_8,X_7,X_6, X_2,X_1, arginine, tyrosine, valine	X_4, leucine, phenylalanine			gluconic acid
	S. enter.	X_9,X_8,X_6,X_2,X_1, proline, asparagic acid	arginine, methionine	glutamic acid		gluconic acid
spleen	PB8006	X_9, X_2, phenylalanine	tryptophan, valine		X_{12}, X_{13}	
	PB6617	X_9, X_1, lysine	arginine, glutamic acid		X_7	
	S. chol. suis	X_9, tryptophan	arginine, valine, phenylalanine, methionine		X_7	
	S. enter.	X_5, X_1, glutamic acid	methionine, valine, tyrosine		X_{11}	fumaric acid

Intra-peritoneal inoculation dose is applied 0.5ml of each bacterial suspension.
PB8006...$0.1x2^{-5}$mg/0.5ml, PB6617...0.1mg/0.5ml, S. chol. suis...0.1mg/0.5ml,
S. enteritidis...$0.1x2^{-2}$mg/0.5ml

Table 2. Effect of amino acids on the infection of mice with S. paratyphi B

administration of amino acids	mice tested										survived mice	agglutination titer	bact. exam. in liver
phenylalanine	W	W	W	W	W	W	D_1	D_3	D_4	D_6	6/10	320(160)	+
arginine	W	W	W	W	W	D_1	D_3	D_3	D_5	D_6	5/10	640(160)	+
methionine	W	W	W	W	D_1	D_1	D_2	D_3	D_3	D_5	4/10	1280(320)	+
tyrosine	W	W	D_1	D_1	D_2	D_2	D_2	D_3	D_3	D_7	2/10	640(160)	+
valine	W	W	D_1	D_1	D_1	D_1	D_2	D_4	D_6	D_7	2/10	640(160)	+
not administrated	W	D_1	D_1	D_1	D_1	D_1	D_1	D_1	D_2	D_7	1/10	160(0)	+

Remarks : W...weak but look like healthy, D...dead and the number means the dead day. The number out of the parenthesis in the agglutination titer shows the O-agglutination and the number in the parenthesis indicates the H-agglutination.

Table 3. Effect of 2, 4-dinitrophenol and glucose solution on the course of infection of mice administrated arginine with Sal. paratyphi B

experimental group	administration of arginine before infection	days after infection 1 2 3 4 5 6 7 8 9 10 11 12 13 14 (5 6 7 8 9: admupistion of inhibitor)	bacteriological examination of blood	liver	spleen	intes-tine	lymph-node	agglutination titer
not administrate arginine	not administrate	D	+	+	+	-	+	
		sW ..... D	+	+	+	+	+	
administrate arginine	For five days, administrate	W..... W H.....H + - + - - - W ..... W H.....H - - - - - +	+	+	+	-	+	640
			+	+	+	-	+	
administration of 2,4-dinitrophenol	for five days administrate	W..... W H.....H - - - - + - W..... W H..... H - - - - - -	+	+	+	-	+	640
			-	+	+	-	+	
		H..W..... sW sW W.... W H..... H + + - + - -	+	+	+	-	+	
administration of glucose solution	for five days administrate	H..W.....W sW sW W.... W H.....H - + - - - +	-	+	+	+	+	640
		W..... W sW sW W..... W H.....H - + + - - -	-	+	+	+	+	
		W.....W sW sW W..... W H....H - - + + + +	+	+	+	+	+	
		W.....W sW sW W..... W H.....H + - - - - -	+	+	+	+	+	
		W.....W D - +	+	+	+	+	+	

H...healthy status, W...weak, sW...severe weak, +...bacteria identified, −...not identified, D...dead

Table 4. Pathogenicity of the original strain and the mutant which require arginine

strain \ dose	0.1mg	0.1×2^{-2}	0.1×2^{-4}	0.1×2^{-6}
original strain PB8006	D_1 D_1 D_1	D_1 D_1 D_1	D_1 D_1 D_1	W W W
mutant strain	D_1 D_1 D_1	D_3 W W	D W E	H H H

Remarks: D....dead and dead day, W....weak, H....healthy

Table 5. Effect of arginine on the oral infection of mice with the original strain (PB8006) and the mutant of Sal. paratyphi B which require the arginine

experimental group		strain challenged	fate of mice	bacteriological examination of stool	blood	liver	spleen	intestine	lymphnode	agglutination titer
administration of arginine before infection	do	original	0/5 (sW 1)	+	-	+	+	+	+	10
		mutant	2/5 (sW 3)	+	+	+	+	+	+	160
		mutant*	1/5 (sW 4)	+	+	+	+	+	+	80
	do not	original	0/4 (sW 0)	+	-	-	-	+	-	20
		mutant	0/4 (sW 0)	+	-	-	-	+	-	10

Remarks: *....the mice were given arginine again after the infection.
sW...severe weak condition

Ⅱ. *The hemolysis of Escherichia coli* (Nagasaki Medical Journal, 32: 1249–1258, 1957, 33: 1–11, 174–181, 1958).

In spite of no evidence on the relation between the pathogenicity and the hemolysis of Escherichia coli, an interest was placed on the study of this problem. The hemolytic E. coli was isolated frequently from the inflamatory location of the appenditis in man. First of all, in this study, the properties of hemolysis of E. coli was studied. The hemolysis of red blood cells of human, horse, bovine, swine, sheep, goat, rabbit and chicken was induced in the culture fluid of E. coli. The hemolytic activity was increased in the presence of tyrosine and vitamin B_1 or yeast extract in the synthetic medium. The hemolysis effect produced in the culture fluid was observed even by the removal of bacteria. The crude preparation of the hemolytic factor could be extracted from the culture fluid by the isoelectric method. The properties of hemolysis was labile to heat and formalin, diffusive, not dialysable and not sedimented by the high speed centrifugation.

Table 6. Biochemical properties of isolates

	number of strain										
appenditio origin	32 33 34 37	42 58		40 44 46	27 47	30 55	48 53 57	28 49	29	54 55 65 66	35	59
fecal origin	5 10 12 20	22 24	13 15		4 16		11	9 17	14 19			
Indol	+	+	+	+	+	+	+	+	+	+	+	+
H_2S production	−	−	−	−	−	−	−	−	−	−	−	−
Urea	−	−	−	−	−	−	−	−	−	−	−	−
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+
V P	−	−	−	−	−	−	−	−	−	−	+	±
M R	+	+	+	+	+	+	+	+	+	+	−	±
Ammonium glucose	+	+	+	+	+	+	+	+	+	+	+	+
Ammonium citrate	−	−	−	−	−	−	+	−	−	−	+	+
Sodium citrate	−	−	−	−	−	+	+	+	+	+	+	+
K C N	−	−	−	−	−	−	−	+	+	+	+	+
glucose	+	+	+	+	+	+	+	+	+	+	+	+
arabinose	+	+	+	+	+	+	+	+	+	+	−	−
xylose	+	+	+	+	−	+	−	−	−	−	−	−
maltose	+	+	+	+	+	+	+	+	+	+	+	+
rhamnose	+	+	+	+	−	+	−	+	−	−	−	−
saccharose	V	−	−	+	+	+	+	−	+	+	+	+
lactose	+	+	+	+	+	+	+	+	+	+	+	+
mannit	+	+	+	+	+	+	+	+	+	+	+	+
salicin	−	−	−	−	−	V	+	−	−	+	+	+
sorbit	+	+	−	+	−	V	−	+	−	−	−	−
dulcit	+	−	−	−	−	+	−	−	−	−	−	−
inosit	−	−	−	−	−	−	−	+	−	−	−	−
asonit	−	−	−	−	−	−	−	V	−	−	−	−
group	E. coli type 1							E. coli type 2			Cloaca	

Remarks: The number of strain with underline indicates the strong hemolysis acitivity.

Table 7. Extraction of hemolysis factor by an isoelectric method

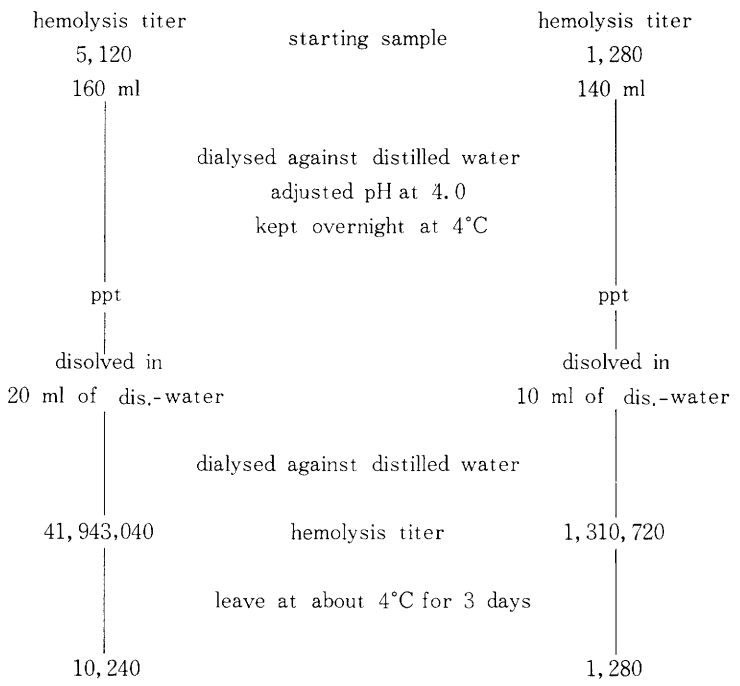

Table 8. The activity of hemolytic factor extracted by the isoelectric method

material	hemolysis titer in experiment of		
	I	II	III
starting material	5,120	10,240	2,280
preparation extracted	655,360	41,943,040	1,310,720
preparation left at 4°C for 3 days	640	20,480	2,560

III. *The experimental infection of mice with rubella virus* (Tropical Medicine, 17: 47-54, 1975).

On the third to the seventh day after mating, when the pregnant mice were challenged with rubella virus by the intramuscular or the intranasal routes, the neutralization antibody was detected in sera of all mother mice and newborns. The virus isolation was performed from the placenta of pregnant mice after two weeks from the virus inoculation. The abnormalities and absorption of fetuses in the uterus of infected mother mice and the high mortality of newborns within two weeks after birth were

observed. It is of considerable value in the study of the virulence of viruses and the transplacental transmission of rubella virus in mice.

Table 9. Incidence of abnormalities and absorption in the uterus of infected pregnant mice with rubella virus

route and day of virus inoculation	number of pregnant mice	day after crossing	fetuses			rate of abnormality
			normal	abnormal	sbsorbed	
non-infected	C1	13	15	0	0	
	C2	15	14	0	0	
	C3	15	13	0	0	
	C4	16	12	0	0	
	C5	16	12	0	0	
total			66	0	0	0%
3 days after intramuscular infection	E1	13	8	2	2	
	E2	13	11	1	1	
	E3	14	9	5	0	
	E4	15	13	0	1	
	E5	15	11	0	0	
total			52	8	4	18.9%
7 days after intramuscular infection	M1	14	6	0	2	
	M2	15	7	0	2	
	M3	15	1	0	0	
	M4	16	12	0	0	
	M5	17	14	0	0	
total			40	0	4	9.1%

Remarks: The rubella virus was inoculated in mice on 3 and 7 day after crossing.

Ⅳ. *The morphology of Japanese encephalitis virus* (Tropical Medicine, 20: 1−14, 1978).

It is considered that the nucleocapsid of alphaviruses is cubic symetry and the similarity of those of flaviviruses is imagined, though the detailed structure of core of flaviviruses is not still determined. Horzineck and Mussgay (1969) described the nucleocapsid of Sindbis virus consisting of 32 polygonal capsomeres within a surface lattice. In contrast, it is not understood clearly the nucleocapsid of flaviviruses.

The author pointed out that the membranous structure could be observed frequently in the fractionated preparation of Japanese encephalitis (JE) virus disrupted by tween 80 and ether and removed the hemagglutinins by the high speed centrifugation after the adsorption with concanavalin A. Furthermore, the double layer form of an empty virion revaled commonly in the preparation of JE virus vaccine. These finding suggest that the nucleic acid, possibly surrounded with proteins, will be enclosed in the membranous

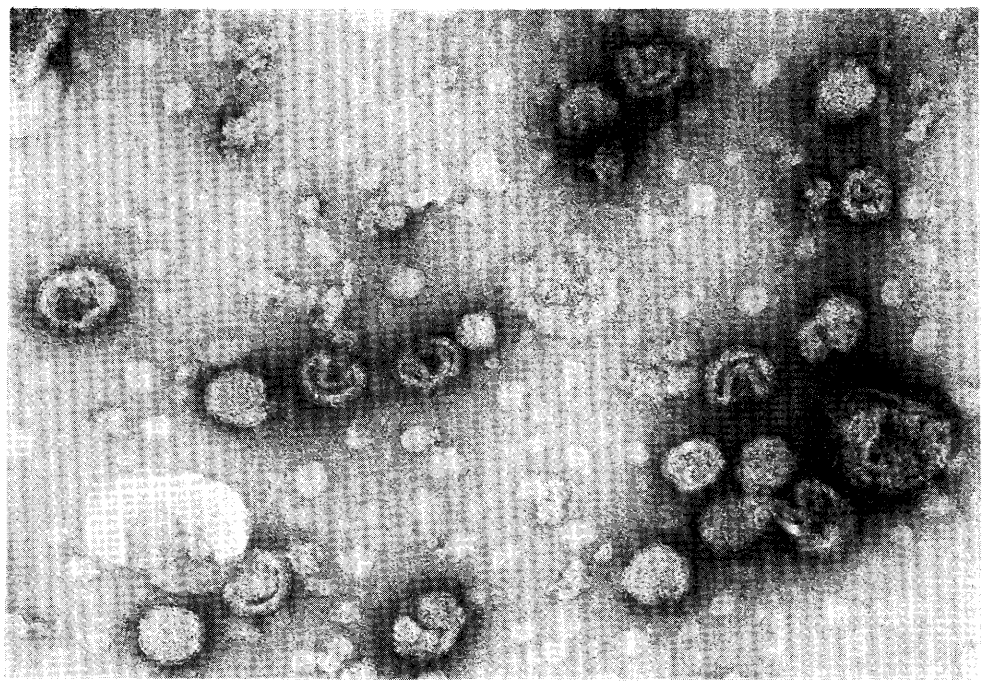

Figure 2. Complete and imcomplete virions of Japanese encephalitis virus. magnification 1:200,000.

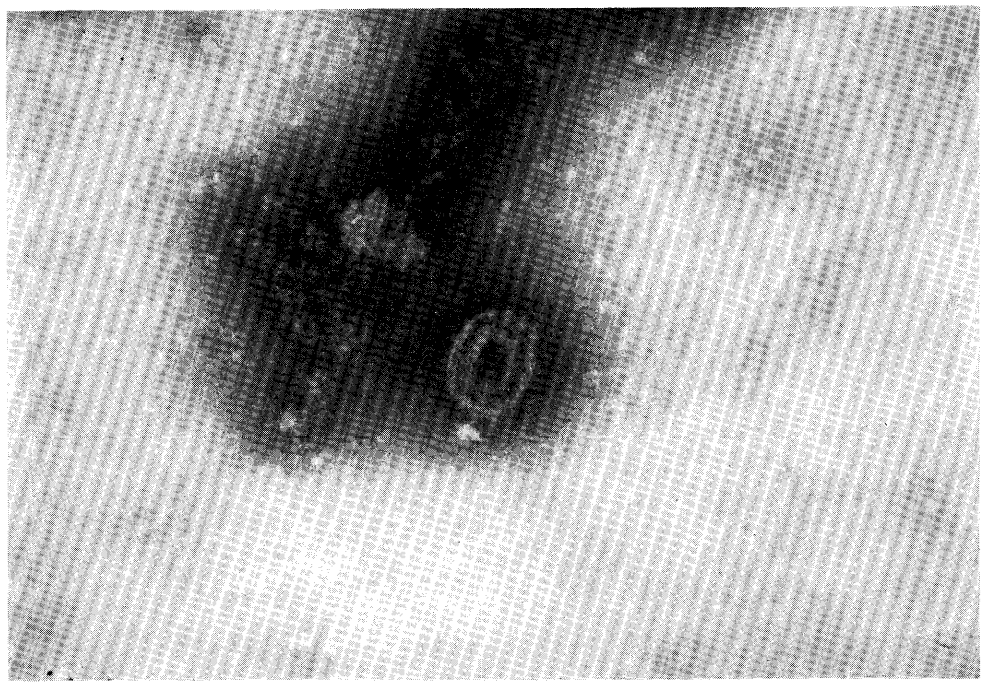

Figure 3. Imcomplete virion of Japanese encephalitis virus. magnification 1:200,000.

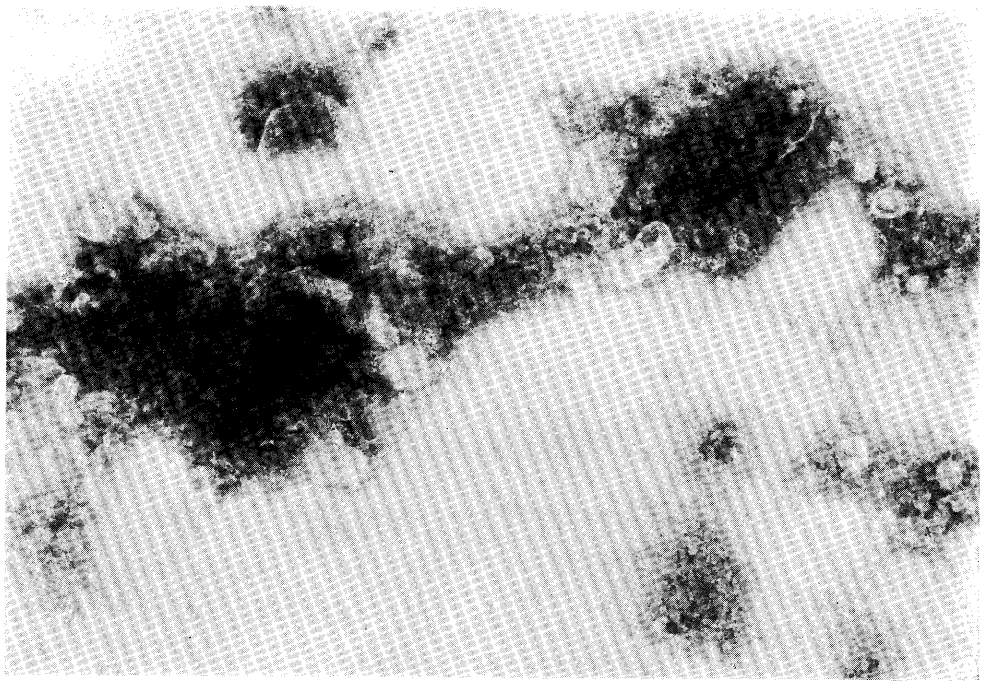

Figure 4. Membranous substances in the fraction separated by density gradient centrifugation from the supernatant removed hemagglutinins by the adsorption of concanavalin A. magnification 1:200,000.

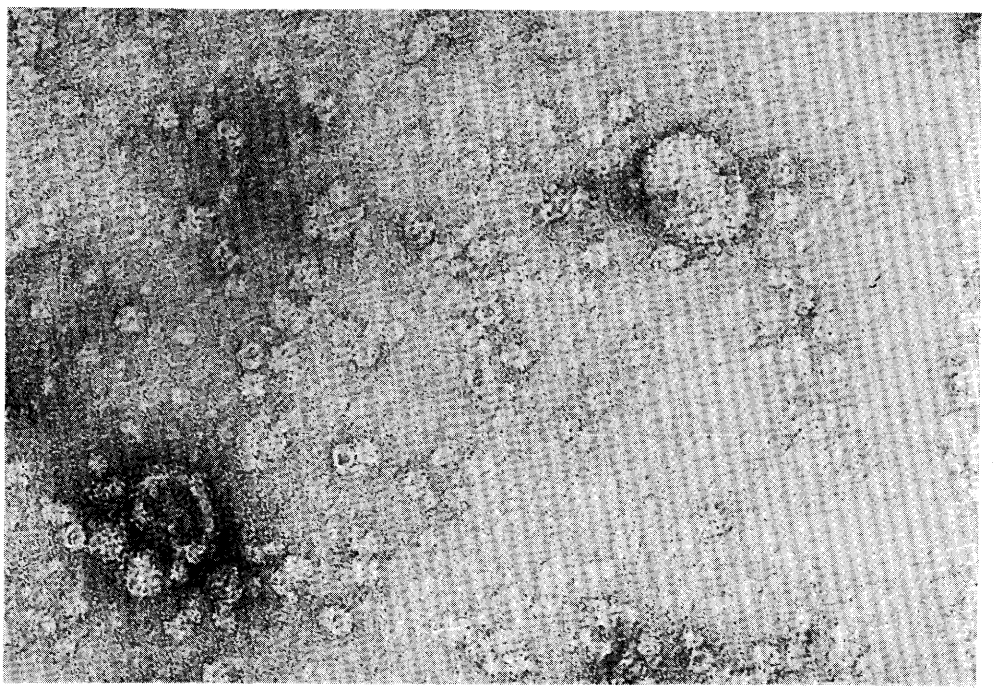

Figure 5. Hemagglutinins separated by the treatment with α-chymotrypsin. magnification 1:300,000.

structure covered with the envelope.

The projections separated from virions induced the antibody related to the neutralization and the hemagglutination inhibition reactions. The small particles having the hemagglutination activity were produced by the connection of several projections. The core component produced the antibody related to the complement fixation reaction.

Table 10. Antigenicity of adsorbed (HAnin) and non-adsorbed (CF-fr) components with concanavalin A

reaction / antigen / antiserum	NT	HI			CF		
	V	V	HAnin	CF-fr	V	HAnin	CF-fr
ascties fluid from immunized mouse with HAnin	64	64	128	< 2	< 2	< 2	< 2
ascites fluid from immunized mouse with CF-fr	< 4	< 4	< 2	< 2	16	< 2	8
ascites fluid from not immunized mouse	< 4	< 2	< 2	< 5	< 2	< 2	< 2

Remarks: NT··· neutralization reaction, HI···hemaglutination inhibition reaction, CF···complement fixation reaction, V···complete virus antigen, HAnin··· hemagglutinaing component, Cf-fr···complement fixing component.

V. *The ecology of Japanese encephalitis virus* (Tropical Medicine, 17: 97−110, 111−127, 129−142, 1975, 17: 159−176, 1976, 20: 81−96, 1978).

Since 1964, the succeeded investigation on the ecology of JE virus is carried out in our laboratory. As well known, JE virus spread widely in the east part of India, the south east Asia, Phillipine, Indonesia, Korea, Japan, possibly China and some of islands in the Pacific Ocean. In Japan, the virus is disseminated with regurality in the summer period every year, even the vector mosquitoes appear from the early spring to the autumn. In the epidemic season, the virus is amplified by the susceptible swine provided in the great scale for the mass cultivation. On the other hand, the virus disappears in nature in Japan from the autumn to the next early summer. It is considered that in the earliest epidemic period, the small focus forming will be made in some area in Japan island and the focus become spread widely. However, it is beyond our knowledge that who gives the virus to the vector mosquitoes in the first focus imagined.

There are some consideration on the overwintering of JE virus in Japan. (1) The carried in theory suggests that migrated and infected birds will transport the virus. The other speculation, which the infected vector mosquitoes might be transported with the wind, is proposed.[2] (2) The persistent theory suggests that the virus will be maintained in nature by keeping the life cycle. It was demonstrated that the experimentally infected vector mosquitoes could survived from the autumn to the next spring and they could transmitted the virus to the susceptible swine. It was also reported that the virus could survived in bats and lizards infected. Recently, Rosen et al. (1979, 1980) demonstrated

Table 11. JE virus isolation from *Culex tritaeniorhynchus* and human JE cases in Nagasaki from 1964 to 1981

year	JE virus from *C. tritae.*		human encephalitis cases			
	isolation period (days)	number of isolates	number of cases reported	dead	comfirmed	period
1964	Jun. 8 - Aug. 7 (61)	19	45	20	n.t.	Jul. 3 - Sep. 10
1965	May 30 - Sep. 6 (100)	47	68	22	34	Jul. 5 - Oct. 23
1966	Jun. 21 - Aug.27 (68)	71	127	54	33	Jul. 2 - Nov. 26
1967	Jun. 23 - Jul. 27 (35)	25	43	21	11	Jul. 18 - Sep. 7
1968	Jul. 18 - Aug.21 (35)	18	20	12	2	Jul. 5 - Sep. 30
1969	Jul. 9 - Aug.28 (51)	20	19	12	4	Jul. 22 - Sep. 26
1970	Jul. 15 - Aug 31 (48)	27	17	11	3	Jul. 23 - Sep. 18
1971	Jul. 12 - Aug. 24 (44)	8	3	3	0	Aug. 8 - Sep. 15
1972	Aug. 16 - Sep. 9 (25)	2	1	0	0	Aug. 10
1973	Jul. 9 - Aug. 13 (36)	19	6	2	4	Jul. 30 - Sep. 15
1974	Jul. 29 - Aug. 12 (15)	3	0	0	0	
1975	Jul. 14 - Aug. 25 (43)	16	1	1		Jul. 19
1976	Jul. 12 - Aug. 9 (29)	7	0	0	0	
1977	Aug. 1 - Aug. 17 (17)	13	0	0	0	
1978	Jul. 10 - Jul. 24 (15)	6	9	6	7	Jul. 31 - Sep. 5
1979	Jul. 3 - Aug. 13 (42)	18	4	1	2	Aug. 25 - Sep. 9
1980	Jul. 28 - Sep. 1 (36)	17	2	1	2	Aug. 28, Sep. 16
1981	Jul. 27 - Aug. 31 (31)	27	1	1	1	Aug: 30

Table 12. Comparative investigation on the virus isolation from vector mosquitoes and the infection of swine

year	first virus isolation from mosqutioes	first detection of 2ME sensitive antibody in swine sera	days of differenec
1964	Jun. 8	-	-
1965	May 30	Jun. 13	15
1966	Jun. 21	Jul. 4	14
1967	Jun. 23	Jun. 24	1
1968	Jul. 18	Jul. 20	3
1969	Jul. 9	Jul. 10	1
1970	Jul. 15	Jul. 22	8
1971	Jul. 12	Jul. 13	1
1972	Aug. 16	Aug. 31	16
1973	Jul. 9	Jul. 13	5
1974	Jul. 29	Aug. 6	9
1975	Jul. 14	Jul. 15	1
1976	Jul. 12	Jul. 13	1
1977	Aug. 1	Aug. 8	8
1978	Jul. 10	Jul. 12	3
1979	Jul. 3	Jul. 10	8
1980	Jul. 28	Aug. 5	9
1981	Jul. 27	Aug. 4	9

Table 13. Whereabouts of Japanese encephalits virus

I. Dissemination of JE virus in the epidemic season
period: May-September ····· Okinawa
July-August ····· Kyushu and main islands
amplification:
mosquitoes - pigs - mosquitoes - ·····
II. Overwintering problem of JE virus
period: October - next April·····Okinawa
September - next June····· Kyushu and main islands
1. persistence theory
successfully experimental infection:
(1) vector mosquitoes of *Culex tritaeniorhynchus*
(2) lizzard
(3) bat
(4) transovarial transmission
2. carried in theory
(1) migrated birds
(2) transportation of infected mosquitoes with the wind
evidences of survey which are suspected the theory
(a) no virus isolation from hibernated female vector mosquitoes
(b) the virus isolation from vector mosquitoes in winter could be performed only in 1973 in Amami island and in 1976 in Okinawa island
(c) the vector mosquitoes could be caught even in small number, on the sea of East China Ocean.
(d) in June and July, a great number of butterflies and some other insects were transported with the wind every year from the south areas to Kyushu island and some places faces to Pacific Ocean in the main island of Japan.

the transovarial transmission using several species of mosquitoes inclnding *Culex tritaeniorhynchus*. However, these experimental results are not verified in the field survey.

The hibernating vector mosquitoes of *Culex tritaeniorhynchus* could be caught effectively in the early spring by the evaporation of dry ice in the field. An attempt to isolate the virus from them was performed unsuccessfully and at the same time, the virus could not isolated from other animals and insects. On the other hand, there were particular evidences that in the early part of February in 1973 and 1976 in Amami and Okinawa islands respectively, the virus could be isolated from resting vector mosquitoes in winter. Except these two incidences, the virus could not detected in nature from the autumn to the next early summer. Such evidences suggest that the virus will be carried in Japan island by something every year. In fact, it is well known fact that some kind of butterflies, which were identified as the south origins, were transported with the wind to the south coast areas of Japan island faced to the Pacific Ocean in the early part of summer season every year.

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マウスの細菌およびウイルス感染における2，3の問題と日本脳炎ウイルスの生態学

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著者は1983年4月1日，退官に際して，1958年以来，公表された論文の中で，特に興味ある課題について述べた．その1つは，マウスのパラチフスB菌感染におけるアルギニンの役割である．感染前のアルギニン投与はマウスのパラチフスB菌感染を停滞させるが，高張糖液の投与はこの停滞を破ることを示した．またアルギニン要求菌の菌力は原株に著しく劣るが，逆に感染前アルギニン投与マウスには原株より著しい菌力の増強を示した．その2は，大腸菌の溶血性である．溶血性は培養液中に存在し菌体を除いても証明される．この物質は等電点法で抽出されるが比較的不安定であった．溶血物質と病原性との関連は明かでなかった．その3は，風疹ウイルスの妊娠マウスにおける垂直感染の証明である．胎内胎児の状態を指標とすることによって，ウイルスの病原性の研究への応用が期待された．その4は日本脳炎ウイルスの形態学に関するものである．ウイルス粒子を Tween 80とエーテルで破かいし，コンカナバリンAで血球凝集素を吸着除去した分画の中に，膜様構造物を認めた．また，ワクチン標本の中にはしばしば二重膜構造の empty virion を認めることから粒子の構成について言及した．その5は，日本脳炎ウイルスの生態学について述べた．日本では流行期における日本脳炎ウイルスの撒布の事情はかなり明かにされてきたが，ウイルスの越冬については全く不明である．ここには今日までの調査成績をまとめて参考とした．

熱帯医学 第24巻 第4号 187－201，1982年12月