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FUJITA, Nobuya

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RESEARCH ON DENGUE IN TISSUE CULTURE

IV. Serologic Responses of Human Beings to Combined Inoculations of Attenuated, Tissue-Cultured Type I Dengue Virus and Yellow Fever Vaccine*

Nobuya FUJITA, Katsuhiko ODA,
Yoshio YASUI** and Susumu HOTTA

Department of Microbiology
Kobe University School of Medicine

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Nobuya FUJITA, Katsuhiko ODA, Yoshio YASUI and Susumu HOTTA. *Research on Dengue in Tissue Culture. IV. Serologic Responses of Human Beings to Combined Inoculations of Attenuated, Tissue-Cultured Type 1 Dengue Virus and Yellow Fever Vaccine.* Kobe J. Med. Sci. **15**, 163-180, December 1969——Using a human-attenuated, tissue-cultured type 1 dengue virus (D1, Mochizuki strain) in combination with the yellow fever (YF) 17D virus vaccine, human immunization experiments were carried out as an extension of the previous work (reported in this JOURNAL, **12**, 199, 1966).

Preliminary data indicated that D1 virus multiplied in trypsinized dog kidney cell cultures. Degree of CPE in the infected cultures was slight. Specific hemagglutination-hemadsorption with goose erythrocytes, however, was positive.

Adults who were residing in the main islands of Japan and who had never entered dengue or YF-endemic areas were divided into the following 5 categories: (A) those inoculated with live D1 and YF viruses simultaneously; (B) those inoculated, first with D1 virus, and two weeks later with YF virus; (C) those inoculated, first with YF virus, and two weeks later with D1 virus; (D) those inoculated with D1 virus, alone, which had been cultivated in Japanese monkey (*Macacus fuscatus*) kidney cell cultures; and (E) those inoculated with D1 virus, alone, cultivated in dog kidney cell cultures. Two shots for each virus were given at 10-week intervals. No abnormal clinical signs were recognized after the inoculations. Serum was taken from each subject and neutralization (NT) and hemagglutination-inhibition (HI) antibodies were measured. Fractionation of serum by sucrose density gradient centrifugation (Suc-DGC), together with a 2-mercaptoethanol (2-ME) treatment were also performed.

Both anti-D1 and anti-YF NT and HI antibodies were effectively produced after combined inoculations of the two viruses. At the same time, the following tendencies were noted: (i) When the D1 and YF viruses were inoculated simultaneously, titers of anti-D1 antibodies were somewhat lower than those produced after inoculating D1 virus alone; (ii) the similar aspect was shown in the subjects inoculated first with YF virus and two weeks later with D1 virus; and (iii) in the subjects inoculated first with D1 virus and two weeks later with YF virus, titers of anti-D1 antibodies were of the same magnitude as those produced by inoculating

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** Present address: Department of Psychiatry and Neurology (Director: Prof. Shoshiro Kuromaru), Kobe University School of Medicine.

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Authors' names in Japanese: 藤田宣哉, 小田克彦, 安井祥雄, 堀田進

D1 virus alone. It appeared, at least under the conditions studied, that titers of antibodies against virus given afterward were lower than those against virus given previously.

Intracutaneous inoculation of comparatively small doses of live D1 virus cultivated in dog kidney cell cultures provoked production of anti-D1 antibodies of significant titers. General patterns of such antibody production were essentially the same as those by the same virus of monkey kidney cell culture origin.

Serum specimens taken at 5 weeks after the first virus shot (i. e., taken at the "first peak" of antibody rise) and those at 12 weeks (i. e., at the "second peak" of antibody rise after the booster shot) were subjected to Suc-DGC and 2-ME treatment. It was indicated that major portions of the antibodies detectable at 5 weeks corresponded to 19S globulin and were ME-sensitive whereas those at 12 weeks corresponded to 7S globulin and were ME-resistant.

The majority of the sera tested had anti-Japanese encephalitis antibodies which, however, apparently showed no direct correlation with the anti-D1 and anti-YF antibodies detected in the present study.

INTRODUCTION

It was previously reported that an attenuated, monkey kidney tissue-cultured strain (Mochizuki) of type 1 dengue (D1) virus stimulated humans and monkeys to produce specific anti-D1 neutralizing antibodies when injected intracutaneously in comparatively small doses of the living state.[3] In this paper results of further studies in which the above strain of virus was given human subjects in combination with the 17D yellow fever (YF) vaccine will be described. Additional experiments concerning fractionation of immune globulins were performed to clarify some aspects of the anti-viral immunity.

MATERIALS AND METHODS

Virus

The D1 Mochizuki strain virus was cultivated serially in Japanese monkey kidney cell cultures. The same virus cultivated in dog kidney cell cultures was also used.* Infected culture fluid was spun by low speed centrifugation, and the

* Technical specifics of dog kidney cultures were essentially the same as those previously reported.[2] The kidney tissues were taken from 1-week-old puppies. Culture medium consisted of 0.5% lactalbumin hydrolysate and 5% inactivated bovine serum in Hanks' balanced salt solution, supplemented with 100 u/ml penicillin and 100 γ/ml streptomycine. Maximum titers of virus in the fluid phase (intracerebral LD_{50}'s in 2 to 3-week-old mice) were 10^5 to 10^6/0.02 ml at about 5 to 7 days after inoculation. CPE was not marked; TCHA (tissue culture hemagglutination-hemadsorption) was, however, positively observed. Procedures of TCHA were as follows: (1) Suck infected cultures; (2) wash gently the cells with Hanks' solution 2 times; (3) add borate buffer solution (BBS, pH 9.0; 2 ml per 150 ml bottle), and hold it at 37°C for 30 minutes, immersing the cells in the fluid; (4) add 1 ml of 1% goose erythrocyte suspension (in BBS including 0.85% NaCl, pH 6.2-6.4), and hold it at 37°C for 1 hour; (5) remove gently the erythrocytes with a capillary pipette as completely as possible; (6) wash gently the cells with phosphate buffer solution (PBS, pH 7.2); and (7) observe *in situ* under a light microscope of low magnification (×5 to 100). Methanol or formalin-Ringer fixation followed by the May-Grünwald-Giemsa stain can be applied.

supernatant was used as inoculum.

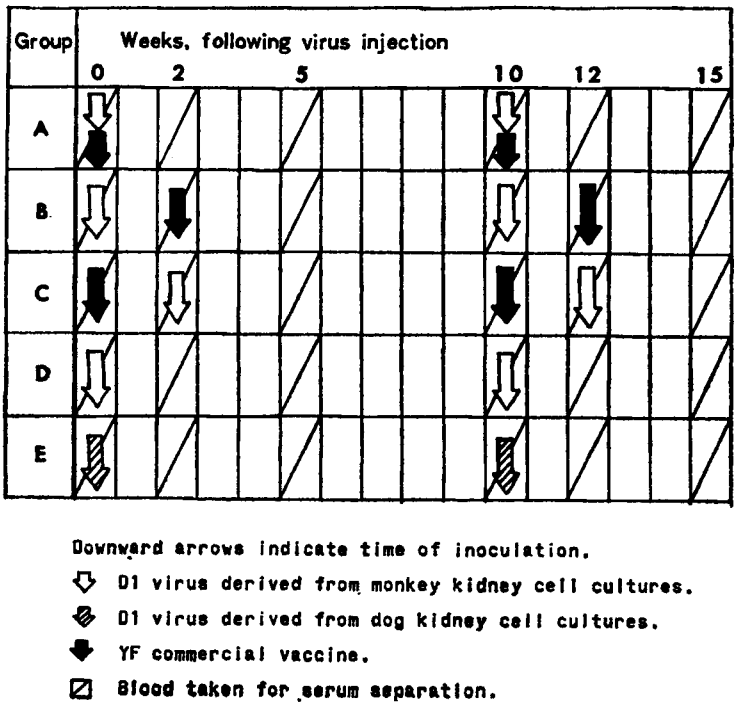
YF virus used was a commercial desiccated 17D vaccine (procured from the National Drug Co., U. S. A.) which was dissolved in sterile distilled water just prior to use.

For NT and HI tests, the same virus strains passed in the brain of mice were employed.

Human subjects

Male and female adults, ranging from 32 to 64 years of age, residing in the main islands of Japan, were subjects of investigation. No one of them had suffered from dengue or YF or had entered dengue or YF-endemic areas prior to the present experiments. They received 0.3 ml live D1 virus intracutaneouly (0.1 ml each at 3 sites) at the flexor side of the right upper arm, and 0.25 ml YF vaccine subcutaneously at one site of the flexor side of the left upper arm. At intervals after the injection, serum was taken from each subject for serologic tests. Schedules of virus injections and serum separations are indicated in Fig. 1.

Fig. 1. Schedules of virus inoculation and serum separation.



Titration of NT antibody

Sera were inactivated by heating at 56°C for 30 minutes and stored at −20°C until ready for use. An accessory factor[10] was not added. The serum,

diluted 5-fold in Hanks' solution, and the original mouse passaged virus, diluted 10-fold serially in the same diluent, were mixed in equal volume. The mixture was kept at 37°C for 1 hour, and in ice water for additional 30 minutes. One fiftieth ml of the mixture was then injected into 2 to 3-week-old mice intracerebrally. Bovine serum (Difco) free from D1 and YF antibodies or inhibitors served as control. Neutralization indices (NI's) were calculated from differences of mouse LD_{50}'s between test and control sera, applying the Reed-Muench formula.

HA and HI tests

Hemagglutinins of both D1 and YF viruses were prepared from infected mouse brains by protamine sulfate treatment according to the previous descriptions from this laboratory.[5] Serum was treated by aceton extraction and goose erythrocyte adsorption by Clarke and Casals' method.[1] HA and HI titers were determined by Takatsy-Sever's microtiter techniques,[18] using U type trays. Of virus adjusting diluents, pH for D1 virus was 6.2, and that for YF virus 6.6.

Sucrose density gradient centrifugation (Suc-DGC)

Density gradient columns were constructed in cellulose nitrate tubes (Beckman 1/2″ dia) by putting 0.6 ml each of 7 grade sucrose solutions (10 to 37% by weight) and 0.6 ml of test serum. After being held at 4°C for 16 hours, the tubes were spun at 35,000 rpm for 16 hours at 4°C (using RPS-40 rotors of a Hitachi 55p type centrifuge). By punching a hole at the bottom of tubes, 12 fractions, each consisting of 15 drops, were collected and tested for HI activity.

2-Mercaptoethanol (2-ME) treatment

The aceton-treated or Suc-DGC'd serum was added with equal volume of 0.2M 2-ME in 0.01M PBS (pH 7.2) and mixed thoroughly. After the mixture was held at 37°C for 1 hour in a tightly sealed tube, the seal was taken off and the tube was put in a bath at 37°C for 10 minutes in order to completely evaporate the 2-ME.

RESULTS

NT titers following combined inoculations of D1 and YF viruses

Two series of experiments were carried out, and the results obtained are illustrated in Figs. 2 and 3.

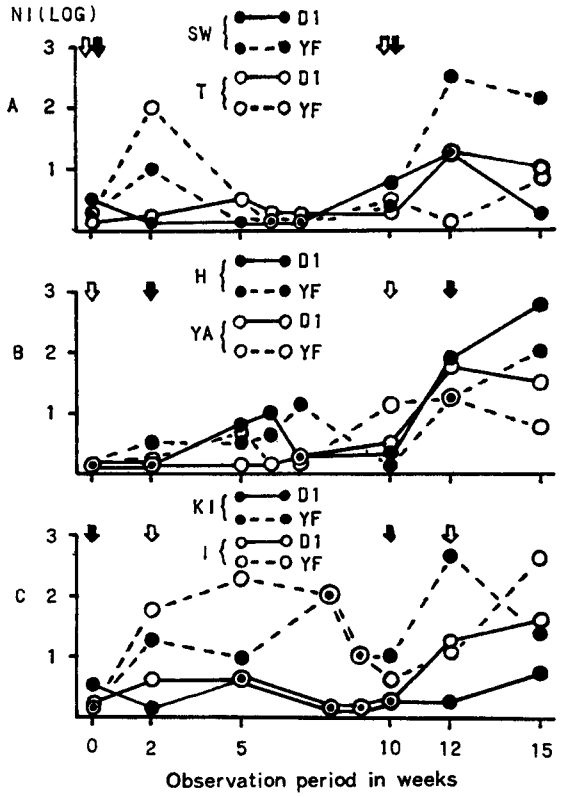
The data apparently indicate that combined inoculations of live D1 and YF viruses induced production of NT antibodies against *both* viruses in the majority of the test cases. In some cases, booster effects after the second inoculation given 10 weeks later were shown.

In addition to these general aspects, the following tendencies were also noted:

(i) When live D1 and YF viruses were inoculated *simultaneously*, titers of anti-D1 NT antibodies were a little lower than those following single D1 inoculations, at least under the conditions studied.

(ii) When the two viruses were inoculated *separately* at an interval of 2 weeks, production of NT antibodies against *virus given afterward* was suppressed to a limited degree.

Fig. 2. Production of anti-D1 and anti-YF NT antibodies in humans following combined inoculations of live D1 and YF viruses (Exp. 1).



D1 virus was harvested from monkey kidney cell cultures and YF virus was a commercial 17D vaccine. Throughout the experiments, viruses of the same lots preserved at −20°C were used. Mouse LD_{50} per 0.02 ml of the D1 virus was $10^{8.5}$, and that of the YF virus was $10^{1.5}$.

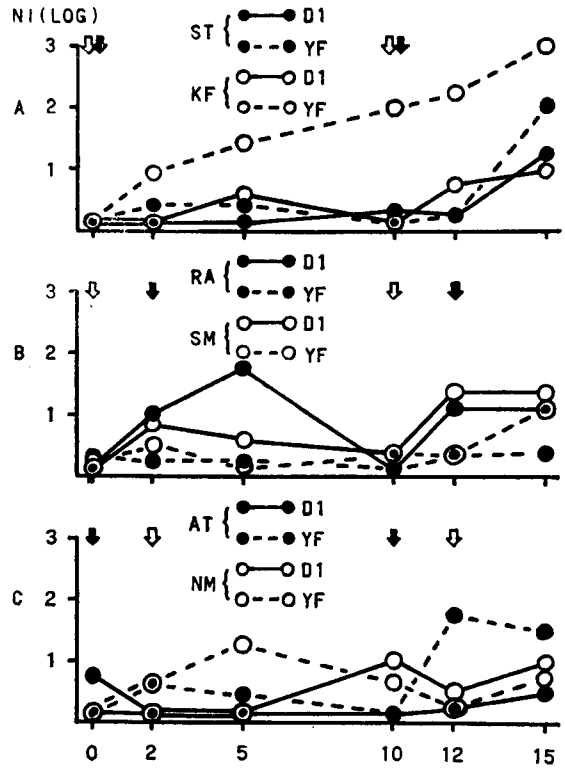
Two adults in Group (A), *SW* (40-year-old male) and *T* (38-year-old female), were inoculated with 0.3 ml of live D1 virus at 3 sites of the right arm, and 0.25 ml of YF virus at one site of the left arm, simultaneously. Ten weeks thereafter, the second shots were given in the same manner. Downward arrows indicate time of inoculation.

Two adults in Group (B), *H* (48-year-old female) and *YA* (33-year-old male), were inoculated, first, with live D1 virus, and 2 weeks later, with YF virus. Ten weeks after the first inoculation, they received the second inoculation similarly.

Two adults in Group (C), *KI* (42-year-old female) and *I* (39-year-old male), were inoculated, first, with YF virus and 2 weeks later, with live D1 virus. Ten weeks thereafter, the second inoculation was made similarly.

Ordinate indicates NI expressed by logarithmic number, and abscissa indicates period of observation in weeks.

Fig. 3. Production of anti-D1 and anti-YF NT antibodies in humans following combined inoculations of live D1 and YF viruses (Exp. II).



The viruses employed in this series of experiment were from the same lots in the former series.

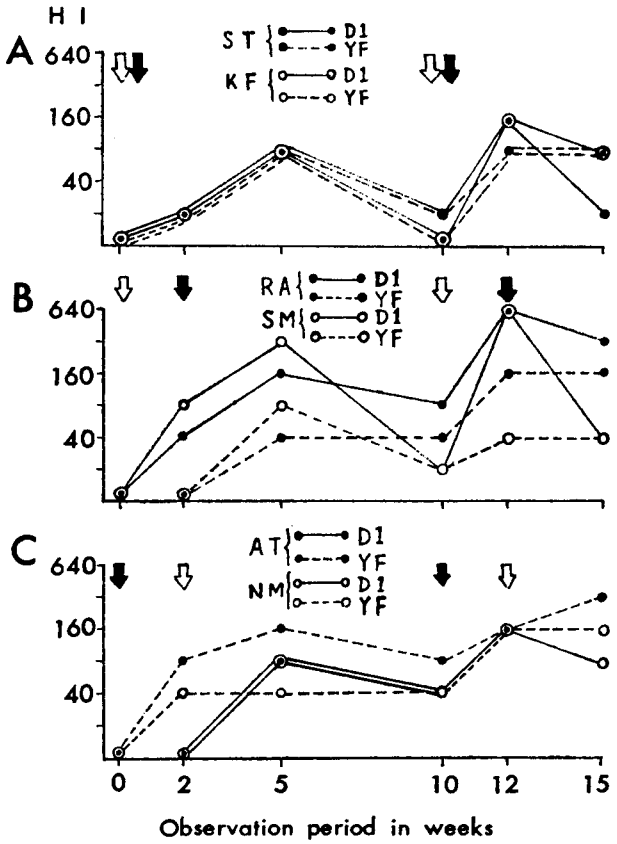
Two adults in Group (A), *ST* (36-year-old male) and *KF* (32-year-old female), were inoculated with D1 and YF viruses simultaneously. Two adults in Group (B), *RA* (43-year-old male)and *SM* (56-year-old female), were inoculated, first, with D1 virus, and 2 weeks later, with YF virus. Two adults in Group (C), *AT* (49-year-old male) and *NM* (49-year-old male), were inoculated, first, with YF virus, and 2 weeks later, with D1 virus.

The conditions of inoculation and other legends are the same as those for Exp. I shown in Fig. 2.

HI titers following inoculations of D1 and YF viruses

Results of HI titrations after single D1 or combined D1-YF inoculations are summarized in Figs. 4 and 5.

Fig. 4. Production of anti-D1 and anti-YF HI antibodies in humans following combined inoculations of live D1 and YF viruses.



Human subjects in this series of experiment are the same ones as those shown in Fig. 3. The conditions of virus inoculations are also the same as indicated in Fig. 3.

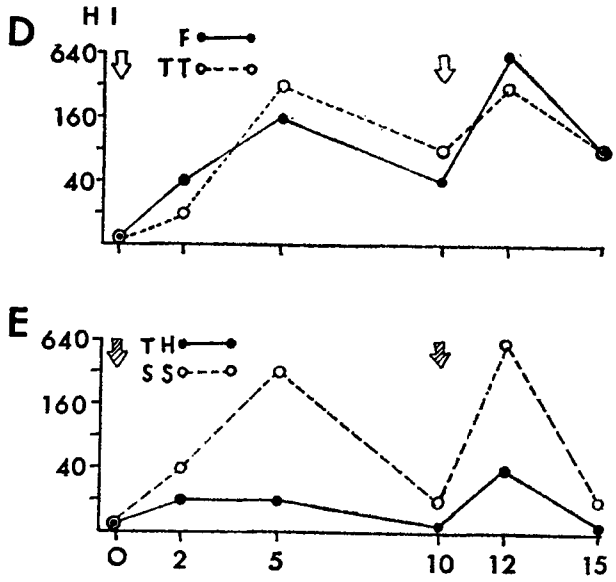
Ordinate indicates HI titers expressed by reciprocals of serum dilutions, and abscissa indicates period of observation in weeks.

It is indicated:

(i) Production of anti-D1 HI antibodies following single inoculations of tissue-cultured attenuated D1 virus (Mochizuki strain) was obvious. Booster effects after the second inoculation were also noted. Titers of antibodies produced by viruses of monkey kidney or dog kidney culture origin were practically the same.

(ii) By combined inoculations of live D1 and YF viruses, HI antibodies against *both* viruses were produced in *all* the cases tested. When, however, the two viruses were inoculated *simultaneously*, anti-D1 titers were a little lower than those obtained by single inoculations of the D1 virus. When the two viruses were

Fig. 5. Production of anti-D1 HI antibodies in humans following single inoculations of live D1 virus cultivated in monkey kidney or dog kidney cell cultures.



Two adults in Group (D), *F* (30-year-old male) and *TT* (57-year-old male), were inoculated intracutaneously with 0.3 ml of D1 virus of monkey kidney cell culture origin, and the other two in Group (E), *TH* (59-year-old male) and *SS* (33-year-old male), were inoculated, similarly as the above two, with D1 virus of dog kidney cell culture origin.

Viruses employed for the first and second inoculations were from the same lots preserved at −20°C; their mouse intracerebral LD_{50}'s per 0.02 ml were $10^{3.5}$ for the monkey kidney culture virus, and $10^{3.25}$ for the dog kidney culture virus.

Other legends are the same as those of Fig. 2.

inoculated *2 weeks apart*, titers of antibodies against *virus given afterward* were lower than those against *virus given previously*.

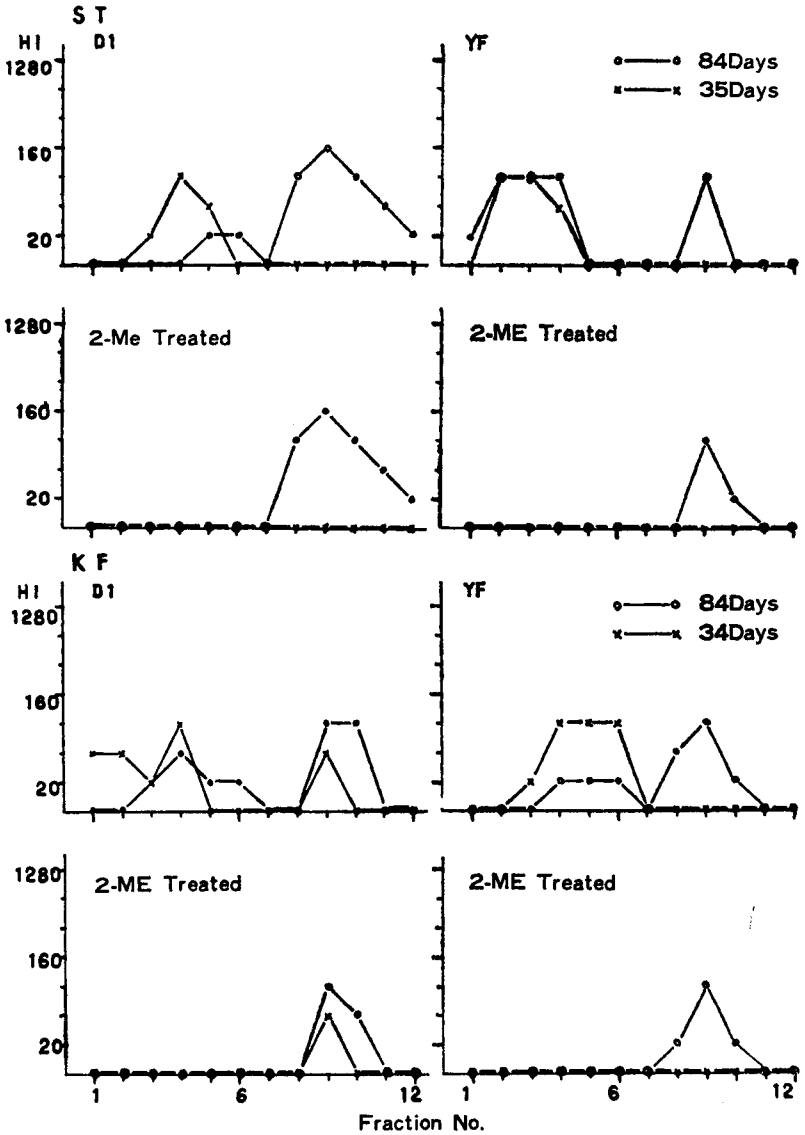
The over-all patterns of HI antibody production are essentially the same as those of NT antibody production as shown in Figs. 2 and 3, as well as those reported in the previous paper from this laboratory.[8]

Suc-DGC and 2-ME treatment of serum

Of the test sera in Figs. 4 and 5, those taken at 34–35 days (i. e., at the "first peak" of antibody rise) and at 84–85 days (i. e., at the "second peak" of

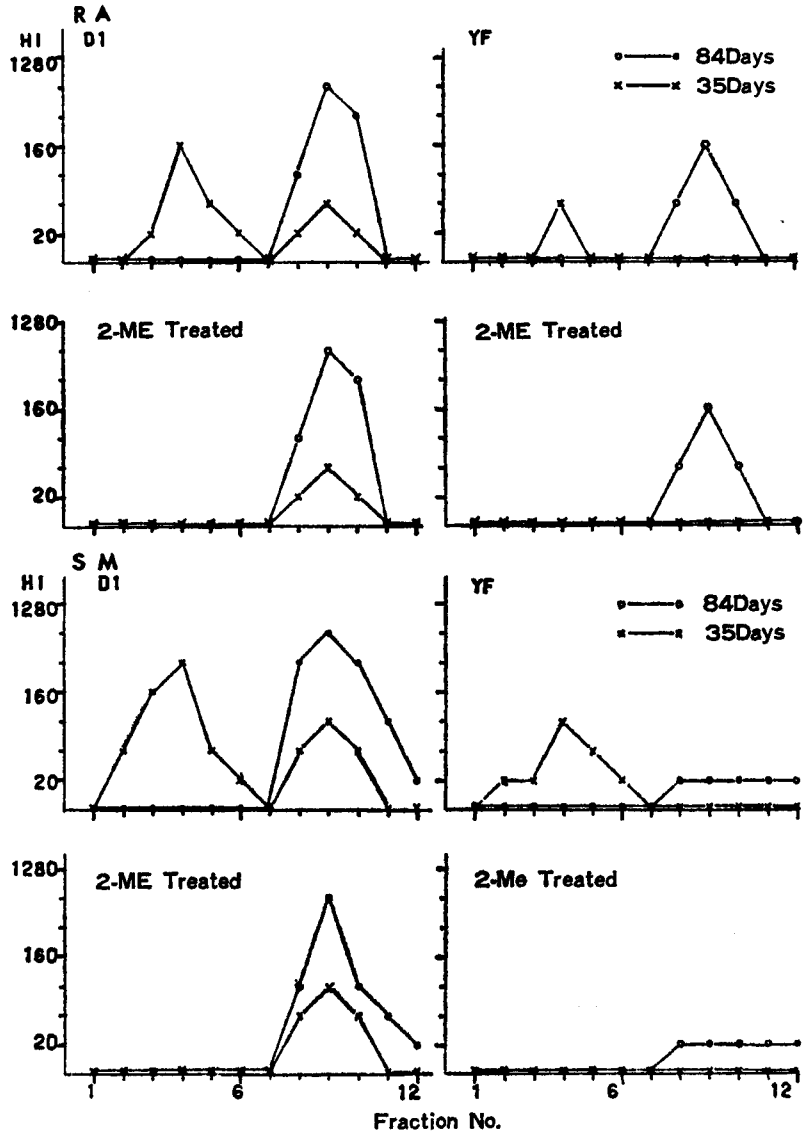
antibody rise) were subjected to fractionation by Suc-DGC together with 2-ME treatment. Results obtained are illustrated in Figs. 6 to 10, and are summarized in Table 1.

Fig. 6. Suc-DGC and 2-ME treatment of serum from human subjects receiving combined inoculations of D1 and YF viruses (Group A : Simultaneous inoculation of D1 and YF).



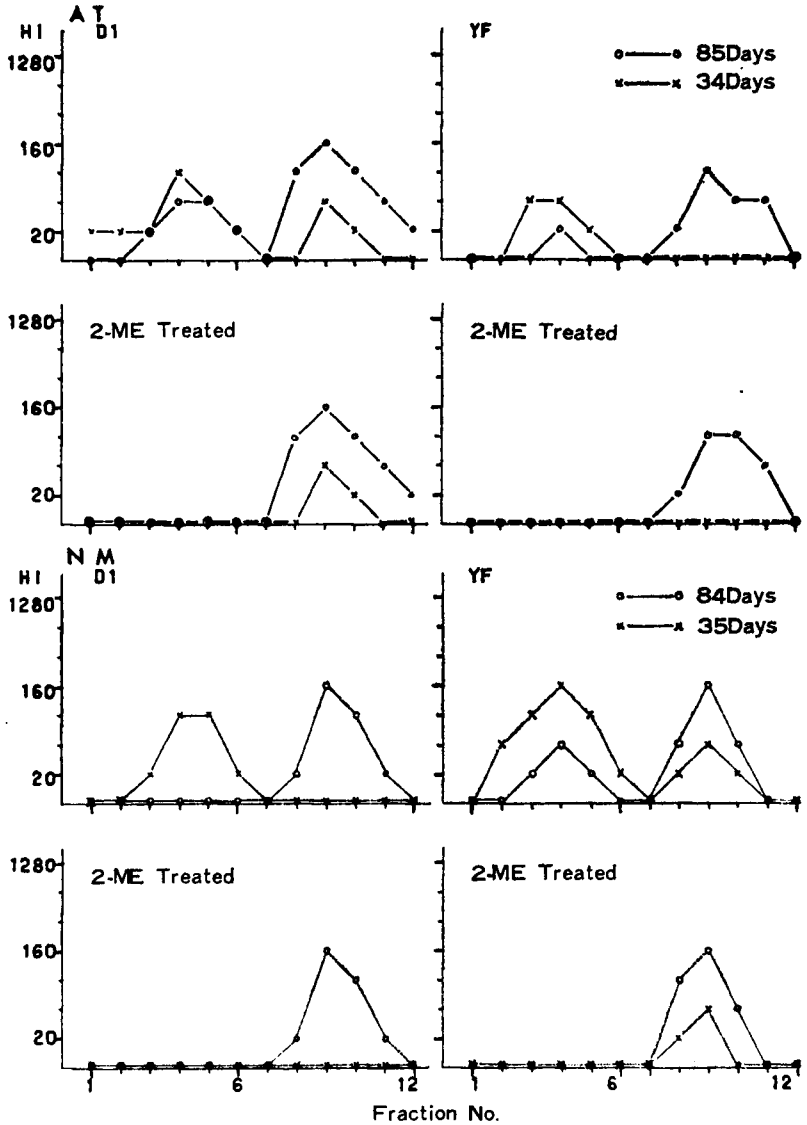
ST : 36-year-old male; *KF* : 32-year-old female (Refer to Fig. 3). Ordinate indicates HI titer. Abscissa indicates number of fractions by Suc-DGC; Fraction 1 is the bottom, and Fraction 12 the top.

Fig. 7. Suc-DGC and 2-ME treatment of serum from human subjects receiving combined inoculations of D1 and YF viruses (Group B : Sequential inoculation, first D1, and 2 weeks later YF).



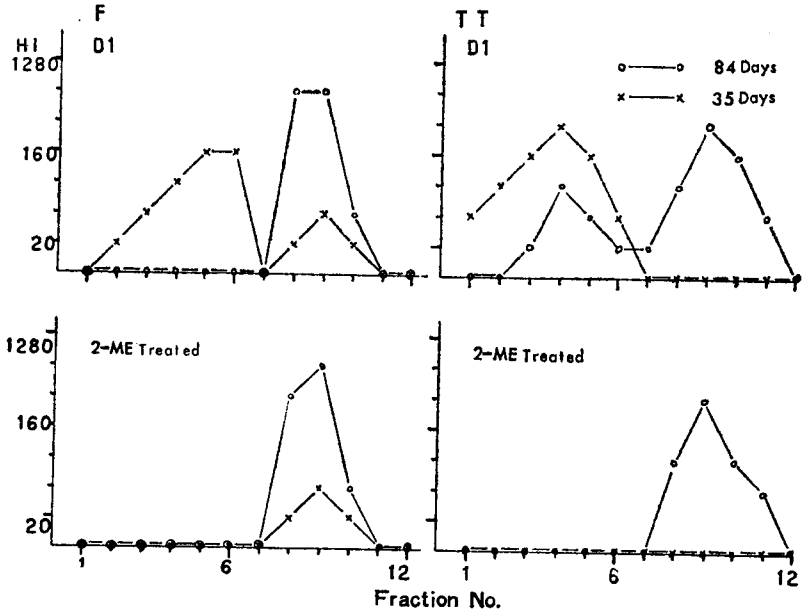
RA : 43-year-old male; *SM* : 56-year-old female (Refer to Fig. 3). Legends are the same as those in Fig. 6.

Fig. 8. Suc-DGC and 2-ME treatment of serum from human subjects receiving combined inoculations of D1 and YF viruses (Group C : Sequential inoculation, first YF, and 2 weeks later D1).



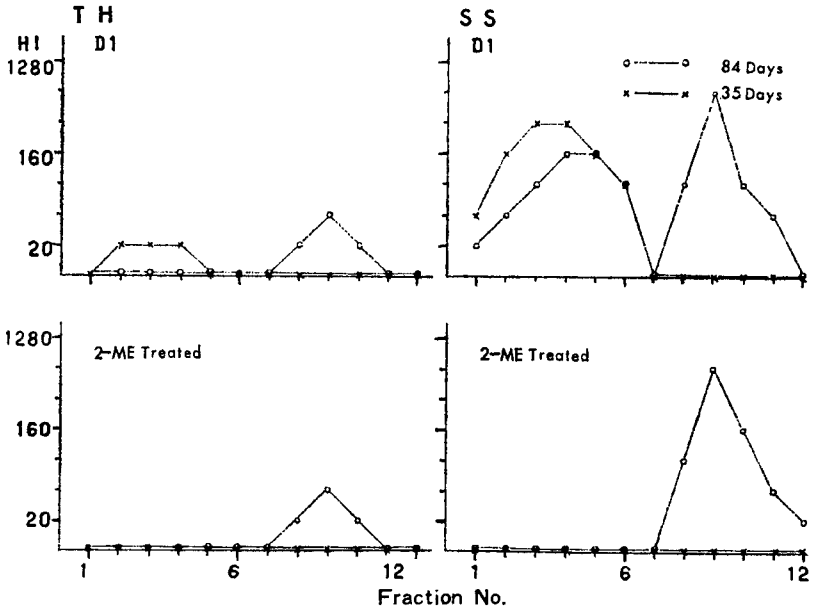
AT : 49-year-old male; *NM* : 49-year-old male (Refter to Fig. 3). Legends are the same as those in Fig. 6.

Fig. 9. Suc-DGC and 2-ME treatment of serum from human subjects receiving single inoculations of D1 virus of monkey kidney culture origin.



F: 30-year-old male; *TT*: 57-year-old male (Refer to Fig. 5). Legends are the same as those in Fig. 6.

Fig. 10. Suc-DGC and 2-ME treatment of serum from human subjects receiving single inoculations of D1 virus of dog kidney culture origin.



TH: 59-year-old male: *SS*: 33-year-old male (Refer to Fig. 5). Legends are the same as those in Fig. 6.

Table 1. Summarized results of HI antibody measurements with serum from human subjects receiving single D1 or combined D1-YF inoculations.

Group	Virus inoculum	Subject	Antibody	Serum taken at 34–35 days		Serum taken at 84–85 days	
				Fraction		Fraction	
				Bottom (ME-sensitive)	Top (ME-resistant)	Bottom (ME-sensitive)	Top (ME-resistant)
A	D1 and YF simultaneously	S T	Anti-D1	+*	−	±	++
			Anti-YF	+	−	+	+
		K F	Anti-D1	+	+	+	+
			Anti-YF	+	−	±	+
B	D1 first, and YF 2 weeks later	R A	Anti-D1	++	+	−	+++
			Anti-YF	+	−	−	++
		S M	Anti-D1	++	+	−	+++
			Anti-YF	+	−	−	±
C	YF first, and D1 2 weeks later	A T	Anti-D1	+	+	+	++
			Anti-YF	+	−	±	+
		N M	Anti-D1	+	−	−	++
			Anti-YF	++	+	+	++
D	D1, of monkey kidney culture origin, alone	F	Anti-D1	++	+	−	+++
		T T	Anti-D1	++	−	+	++
E	D1, of dog kidney culture origin, alone	T H	Anti-D1	±	−	−	+
		S S	Anti-D1	++	−	++	+++

* HI titer : +++≥640; ++=320–160; +=80–40; ±≤20; −=0.
Prior to virus inoculation, neither anti-D1 nor anti-YF HI antibodies were present in all the serum specimens tested.

It is apparent:

(i) that HI activities of serum are separated into two parts by Suc-DGC, i. e., bottom part (rapid-sedimenting fractions), and top part (slow-sedimenting ones);

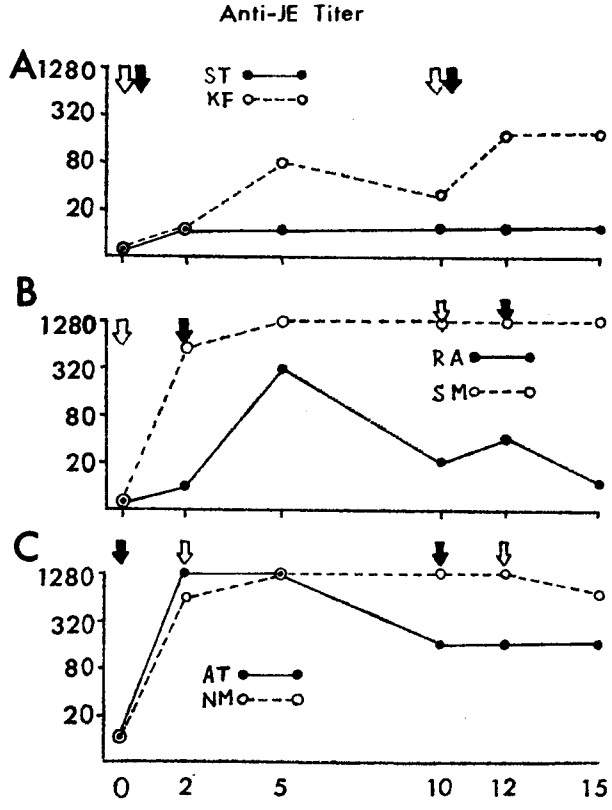
(ii) that the HI activities of serum taken at 34-35 days after the first virus inoculation are mainly in the bottom part; contrarily, those at 84-85 days are principally in the top part; and

(iii) that the activities of the bottom part are lost by 2-ME treatments, and are, therefore, ME-sensitive, whereas those of the top part are ME-resistant.

Anti-JE HI titers following inoculations of D1 and YF viruses

Since Japanese encephalitis (JE) is endemic to the main islands of Japan, it is very probable that many of residents in Japan possess anti-JE antibodies induced by apparent or inapparent JE infection. Parts of the present experiments were carried out during seasons when JE was prevailing, and actually there were typical JE cases in areas where the tested subjects resided at that time. It was considered necessary, therefore, to look into possibilities whether the anti-D1 and anti-YF

Fig. 11. Anti-JE HI antibodies in human subjects receiving combined inoculations of D1 and YF viruses.

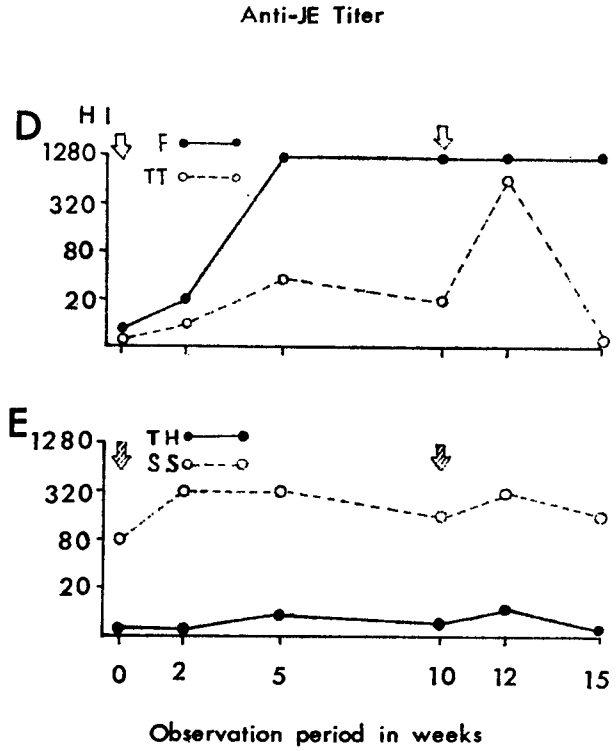


Two adults in Group (A), *ST* and *KF*, received simultaneous inoculations of D1 and YF viruses. Two in Group (B), *RA* and *SM*, were inoculated, first with D1 virus, and 2 weeks later with YF virus. Two in Group (C), *AT* and *NM*, were inoculated, first with YF virus, and 2 weeks later with D1 virus.

As to schedules of inoculation, refer to Fig. 1. Other legends are the same as in Fig. 4.

antibodies as detected in the present tests might be correlated with the anti-JE antibodies. Measurements of anti-JE antibodies (against JaGAr#01 antigen) were conducted from this viewpoint, and the results obtained are summarized in Figs. 11 and 12.

Fig. 12. Anti-JE HI antibodies in human subjects receiving single inoculations of D1 virus.



Two adults in Group (D), *F* and *TT*, were inoculated with D1 virus cultivated in monkey kidney cell cultures, and the other two in Group (E), *TH* and *SS*, with the same virus cultivated in dog kidney cell cultures. Other legends are the same as in Fig. 5.

(i) In four of the test cases (*SM*, *NM*, *F* and *SS*), anti-JE HI antibodies rose after the first virus injection and retained the highest levels until the end of observation periods.

(ii) In two cases (*RA* and *AT*), the antibody titers rose after the first virus injection and decreased gradually thereafter, showing no "booster effect" even after the second D1-YF injection.

(iii) In two cases (*KF* and *TT*), shifts of anti-JE titers showed certain resemblance to those of anti-D1 and anti-YF antibodies induced by the virus injections.

(iv) The remaining two (*ST* and *TH*) exhibited a completely negative HI reaction against JE virus.

It appears that there was no direct correlation between the anti-JE antibodies and the anti-D1 and anti-YF antibodies.

DISCUSSION

As a preliminary account of the present study, data were presented showing that a human-attenuated strain (Mochizuki) of D1 virus retained its immunogenicity for humans after being cultivated in dog kidney cell cultures. Practically no difference was noted in anti-D1 HI titers between cases injected with the virus either of monkey kidney or dog kidney culture origin. This may be of practical significance for development of anti-dengue live vaccine. Breeding of dogs in quarantine is possibly easier, microbiologically and economically, than of monkeys. Therefore, dog tissue cultures may be less hampered by contamination of "latent" agents.

Combined inoculation of live D1 virus and 17D YF vaccine induced production of NT and HI antibodies against *both* viruses. On the other hand, it was shown that *simultaneous* inoculation of D1 and YF viruses brought about what could be considered to be a "suppression" of anti-D1 antibody. Suppressive effects of the apparently similar sort were found in experiments in which the two viruses were injected *2 weeks apart;* titers of antibody against *virus given afterward* were lower than those by single inoculation of the same virus or those against *virus given previously*.

It has been reported by previous investigators[12] that inoculation of human subjects with attenuated, mouse-passaged type 2 dengue (D2) virus in combination with YF vaccine or with YF vaccine plus attenuated, mouse-passaged D1 virus induced predominant production of anti-D2 NT and CF antibodies, namely, suppression of anti-YF or anti-D1 antibodies. The results are compatible, in general tendencies, with the data by the present authors, although relative potencies of the dengue and YF antigens employed by the two groups of investigators were apparently different. A possible explanation of these phenomena may be an "interference" of the viruses. Interference between D1 and YF viruses has been noted in *rhesus* monkeys or *Aedes* mosquitoes[11] or in hamster kidney tissue cultures.[4] A possibility that live dengue and YF viruses "interfere" with each other in the human body under certain conditions must be taken into consideration.

Nevertheless it was evident that NT and HI antibodies against D1 and YF viruses were effectively produced after the combined inoculations of the two viruses. More effective immunization may be expected by considering combination of virus inoculated and order of inoculation. According to Price and his colleagues,[6,7] monkeys inoculated sequentially with certain group B arboviruses, including particular types of dengue, were afforded protection (as judged by degrees of viremia) from challenge with other types of dengue, and showed the highest levels of NT antibodies against heterologous dengue types. Similar possibilities to induce what can be thought as "broad spectrum immunization" against group B arboviruses

by utilizing closely related viruses of the same group as immunogens have been reported (Refer to works by other investigators as cited by the above authors[6,7]).

The results of Suc-DGC experiments indicated that the antibodies detectable early after virus inoculation were of 19S globulin nature whereas in later stages those corresponding to 7S globulin were predominant. The former was ME-sensitive and the latter ME-resistant. While this is not remarkably compared with previous works dealing with other kinds of virus antigens, it is to be mentioned that the present data are indicative of *de novo* production of anti-D1 and anti-YF antibodies after the primary inoculation of viruses. It is also to be noted that the detected anti-D1 and anti-YF antibodies had no apparent correlation with anti-JE antibodies which can be found in many of Japanese residents. According to Russell and his colleagues[9] who examined anti-dengue antibodies in monkeys and humans, antibodies detected at 1-2 weeks after primary infection were predominantly of 19S globulin and those at 3-4 weeks or later were of 7S globulin; antibodies appearing after second dengue infection were entirely of 7S. The same authors[8] also reported that anti-dengue antibody activities in sera from children with dengue shock syndrome were mainly attributable to IgG Fraction I, and they considered the data as a supporting evidence for the hypothesis that this peculiar syndrome is caused by repeated infections of dengue and/or related viruses. It should be stated here that no abnormal clinical signs have been recorded in the present as well as previous dengue immunization studies done in this laboratory, although the number of the test cases is limited.

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