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Artikel: Experiments of active immunization against Dengue with mouse-passaged unmodified virus

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Experiments of Active Immunization Against Dengue with Mouse-Passaged Unmodified Virus.[1]

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Recently, the modified strains of dengue virus have been secured; and the possibility of antidengue immunization with active virus has been suggested (SABIN, 1952; HOTTA, 1952). Since the modification as observed, however, could not be seen through previously by the author, several experiments of active immunization with the unmodified virus were carried out in his laboratory. The results obtained may be still useful for the clarification of the properties of dengue virus, as well as the interpretation of the antidengue immunity. This paper is concerned specifically with the mouse-passaged unmodified dengue virus of the Mochizuki-strain (HOTTA, 1952).

Materials and Methods.

The mouse brain passaged, unmodified dengue virus was pathogenic, although somewhat inconstantly, for mice through the subcutaneous or intraperitoneal route, while with the modified strain, the extraneural infection has encountered a great difficulty. The unmodified virus could be abundantly demonstrated in the blood of mice after an intracerebral infection. In view of these facts, the mixtures of brain emulsion and heart blood from the typically infected mice were chosen as basic ingredients of vaccine, and the viability tests for vaccine were performed through the subcutaneous inoculation in mice. Viral concentration of the materials above was adjusted to be about 10^4 times the mouse-intracerebral LD50 per 0.02 ml.

Inactivation of dengue virus by formalin or ox-bile.

Formalin:

The virus suspensions, as indicated above, were mixed with formalin (35% aqueous solution of formaldehyde) in the ratio of 0.2%, or of 0.4%, respectively, and kept for 2 hours at 37° C. and for 24 further hours at about 10° C. Then, 0.2 ml. of each mixture

[1] Preliminary accounts of part of this paper were published in Japanese by *Kimura* and *Hotta* in 1944 (Jap. J. Bact., *1:* 96-99) and by *Hotta* in 1947 (Nippon Igaku, No. 3413, 87-89).

was inoculated subcutaneously into mice weighing 6 to 7 g. One protocol of the results obtained is indicated in Table 1. The activity of dengue virus was lost to a large extent, but not completely, under the above conditions.

TABLE 1.

Inactivation of unmodified dengue virus by formalin.

Materials	Ratio of addition of formalin (%)	Mice	
		Mortality ratio *	Average incubation ** period, in days
Formalinized	0.4	2/5	12.5
	0.2	2/5	9.5
Control (Non-formalinized)		4/5	7.0

* Denominator: Number of inoculated mice.
Numerator: Number of dead mice.
** Relative to the fatal cases.
These indications will be applied similarly in the following tables.

Ox-bile:

Ox-bile, taken soon after slaughtering, filtered through pulp and sterilized at 100° C., was added to the virus suspensions at the ratio of 10%, 7% or 5%, respectively. The mixtures were kept for 2 hours at 37° C., and for 24 further hours at about 10° C. Then, 0.2 ml. of each mixture was inoculated into the mice subcutaneously. One example of the tests is shown in Table 2, indicating that the dengue virus was inactivated completely by adding ox-bile at 10% or 7%, and incompletely at 5%.

TABLE 2.

Inactivation of unmodified dengue virus by ox-bile.

Materials	Ratio of addition of ox-bile (%)	Mice	
		Mortality ratio	Average incubation period, in days
Bile-added	10	0/5	—
	7	0/5	—
	5	3/5	9.0
Control (Not bile-added)		5/5	10.0

Experiments of active immunization in mice.

Based on the results above, active immunization tests were carried on as follows:

Active immunization of mice with formolvaccine:

Test mice were inoculated, at first with 0.1 ml. of the 0.4% formolvaccine, and secondly, after 5 days, with 0.1 ml. of the

0.2% formolvaccine, both subcutaneously. Mice which died during the immunizing period were rejected from observation. Ten days after the second inoculation, they were challenged with the active virus of 10^3 times the LD50 by the intracerebral route. As a control, non-vaccinated mice were used, that had been maintained under the same conditions as the vaccinated groups during the same period. It should be kept in mind that mice weighing 6 to 7 g. grew up usually to 7 to 8 g. or so during the immunizing period above, and exhibited a decrease of susceptibility or an increase of resistance to the virus, which is probably a consequence of the growth. One example of the protocols is indicated in Table 3. The decrease of mortality ratio and the prolongation of survival period could be evidenced in the vaccinated mice, as compared with those of the control groups. It was concluded, therefore, that the formolvaccine could stimulate in the mice a protecting immunity for dengue infection.

TABLE 3.

Efficacy of antidengue formolvaccine in mice.

Groups	Mice		
	Number of deaths during immunizing period	Mortality ratio	Average incubation period, in days
Immunized	2	5/8	12.0
	2	6/11	13.0
Control	2	7/8	9.1
Immunized	1	8/9	14.3
Control	1	4/4	13.5

Active immunization of mice with ox-bile vaccine:

Mice were inoculated first with 0.1 ml. of the 7% ox-bile vaccine, and next, after 5 days, with 0.1 ml. of the 5% ox-bile vaccine, both subcutaneously. Ten days after the second inoculation, active

TABLE 4.

Efficacy of antidengue ox-bile vaccine in mice.

Groups	Mice		
	Number of deaths during immunizing period	Mortality ratio	Average incubation period, in days
Immunized	1	7/9	15.4
Control	0	7/10	14.9
Immunized	1	10/10 *	14.7
Control	1	4/4	13.5

* One case of non-characteristic death, which occurred 3 days after the challenge, was rejected.

dengue virus of 10^3 times the LD50 was injected intracerebrally into the vaccinated mice, as well as the non-vaccinated controls. One protocol of the results obtained is shown in Table 4, in which little efficacy for protection of the ox-bile vaccine is found.

Vaccination of human beings with formolvaccine or with ox-bile vaccine.

All investigations concerning this subject were conducted in the city of Kyoto, Japan, during the period from February to April, 1944, when there were no possibilities of natural occurrence of dengue, and the human volunteers under observation were isolated in a hospital during the observation periods. The volunteers, prior to the vaccinations, were confirmed to possess no specific neutralizing antibody against the mouse-passaged dengue virus. The technique of neutralization test concerned in this paper was the same as described in the previous reports (HOTTA, 1952; 1953): The serum-virus mixture was kept at 37° C. for 2 hours, at 4° C. for 2 further hours, and then injected into mice intracerebrally.

Vaccination of human beings with formolvaccine.

Two adults (Nos. 1 and 2) were inoculated, first, with 0.5 ml. of the 0.4% formolvaccine, and secondly, after 10 days, with 0.5 ml. of the 0.2% formolvaccine, both into the stretched skin of the upper arms. No abnormal sign was observed either subjectively or objectively, except for a slight induration and a slight pruritis appearing at the injected sites. Three weeks after the second inoculation, the serums were taken out, of which the in vitro neutralization tests with the mouse-passaged virus were performed. Their neutralizing powers, however, were both very slight, as shown in Table 5. Owing to circumstances of that time, no opportunity was afforded to challenge the volunteers with virulent dengue virus of human origin; but it was inferable, judging from

TABLE 5.

Neutralization tests with serums from volunteers inoculated with antidengue formolvaccine.

Serum			Mice	
Volunteers		Concentration	Mortality ratio	Average incubation period, in days
Vaccinated	No. 1	Undiluted	5/5	13.4
	No. 2	Undiluted	4/4 *	14.0
Control		Undiluted	5/5	14.0

* One case of non-characteristic death, which occurred about 30 hours after the injection of serum-virus mixture, was rejected.

the amount of antibody produced, that little protection would have been exhibited thereto.

Vaccination of human beings with ox-bile vaccine.

Four adults (Nos. 3-6) were inoculated, first, with 0.5 ml. of the 7% ox-bile vaccine, and next, after 8 to 10 days, with 0.5 ml. of the 5% ox-bile vaccine, both intradermally. Local reactions found at the injected sites were slight, as of the formolvaccine described above.

Nine days after the second inoculation, however, one of the volunteers (No. 3) showed a temperature-rise of 37.6° to 38.2° C., which persisted 3 days. He also complained of a headache and general fatigue, but no eruption was apparent. The serum, taken 2 weeks after disappearance of the symptoms, was found to contain the specific neutralizing antibody against the mouse-passaged virus. The presumption was, therefore, that he was infected with dengue because of the incomplete inactivation of the virus.

The other three persons were apparently healthy during the 2 to 3 weeks after the second inoculation. The serum from one of them (No. 4), taken 2 weeks after the second inoculation, was found to have no specific neutralizing activity. He fell ill with typical dengue symptoms after being injected intracutaneously with fresh serum from a dengue patient of the febrile stage. (See Table 7, b.)

The other two persons' protocols were as follows:

(a) The serums, drawn out 3 weeks after the second inoculation, were both shown to contain the specific neutralizing antibody. (See Table 6.)

(b) Six weeks after the second inoculation, they were challenged with the virulent human dengue virus by an intracutaneous in-

TABLE 6.

Neutralization tests with serums from volunteers inoculated with antidengue ox-bile vaccine.

Volunteers	Serum	Concentration	Mice: Mortality ratio	Mice: Average incubation period, in days
Vaccinated	No. 5	Undiluted	1/5	10.0
		10-fold diluted *	5/5	12.8
	No. 6	Undiluted	0/4 **	—
		10-fold diluted *	5/5	16.2
Control		Undiluted	6/6	15.2

* With Tyrode's solution.

** One case of non-characteristic death, which occurred 3 days after the injection of serum-virus mixture, was rejected.

jection. One (No. 5) exhibited no particular sign either subjectively or objectively during an observation period of 6 weeks. The other (No. 6) showed a transient temperature-rise above 37° C, as well as general fatigue and a headache which appeared on the 10th day after the challenge, although no conclusive evidence could be obtained about the cause of the symptoms.

A non-vaccinated volunteer who served as control (No. 7) manifested typical dengue symptoms after an intracutaneous injection of the virulent virus from the same lot as for the vaccinated group. (See Table 7, b.)

The main points of these human-experiments are summarized in Table 7 (a, b).

TABLE 7 a.

Results of antidengue vaccination of human beings with formolvaccine or with ox-bile vaccine.

Groups	Volunteers No.	Sex	Age	Safety of vaccine	Production of neutralizing antibody	Infection*	Remarks
Formol-vaccine	1	F	35	Complete	— (3) **		
	2	F	32	Complete	— (3)		
Ox-bile vaccine	3	M	26	Not complete			
	4	M	29	Complete	— (2)	+++ (4) **	See Table 7 b
	5	M	34	Complete	+ (3)	— (6)	
	6	M	32	Complete	+ (3)	± (6)	
Control	7	M	36		—	+++	See Table 7 b

* Degree of the symptoms manifesting after the challenge.

** Parentheses indicate the periods, in weeks, from the second vaccine-inoculation to the examination.

TABLE 7 b.

Progress of dengue infection induced by intracutaneous injection of virulent human serum (Volunteers, No. 4 and No. 7).

Volunteer No.	Incubation period, in days	Fever: Duration in days	Fever: Maximum (C)	Fever: Curve	Symptoms: Headache, lumbago and joint pain	Symptoms: Fatigue and anorexia	Symptoms: Blush of face and skin erupt.	Symptoms: Hemorrhagy or dermal petechia	Leucocyte count (×100)*	Remarks**
4	6	6	39.8	Saddle-form	+++	++	++	—	39/56	M (19)
7	7	10	38.7	Saddle-form	+++	+	++	+	35/57	R.L.

* Denominator: Number before the infection. Numerator: Minimal number during examination period.

** M (19): Monocytosis (19%), on the 2nd day of illness. R. L.: Rumpel-Leede's sign was positive on the 3rd day of illness.

Discussion.

It has been reported coincidently by several investigators that dengue virus, either in the form of human serum or propagated in mosquito or in monkey, loses its immunogenic capacity as it is completely inactivated by physical or chemical agents (BLANC and CAMINOPETROS, 1929, 1930; SIMMONS et al., 1931; ST. JOHN and HOLT, 1931; SABIN, 1948, 1952). Results of BLANC and CAMINOPETROS, however, indicated that their antidengue vaccine, prepared by mixing patient's blood and ox-bile, was capable of protecting human bodies from subsequent challenge with the virulent virus. ISHII (1948) claimed the efficacy for humans of an antidengue formolvaccine prepared from his own virus strain.

In the author's experiments described above, the mouse-passaged unmodified Mochizuki-strain dengue virus, as inactivated by formalin, could stimulate a protecting immunity in white mice, while the virus treated with ox-bile exhibited little efficacy. In regard to humans, however, the formolvaccine was deemed to be without effect, possibly because of a difference of the immunological conditions, such as the species or the relative amount of the vaccine inoculated. The ox-bile vaccine, on the contrary, was shown to be effective in human beings for the antidengue protection to some extent. But it appeared that the virus-inactivating capacity of ox-bile was not always perfect, and therefore the practical application of ox-bile vaccine was not advisable, at least under the conditions described above. It would be probable from these results, moreover, that antidengue immunity could be produced either by a relatively large amount of inactive virus, or by the virus not completely inactivated but attenuated so as to maintain the activity to a certain extent. The latter possibility might be correlated with the deduction that the modified strains of dengue virus could multiply in human bodies without exerting harmful affection (HOTTA, 1952). Since the dengue virus strains so modified have been successfully obtained, antidengue vaccine from the inactive virus may not be necessarily needed.

The immunizing capacity for mice of the modified dengue virus will be discussed in a later report.

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Résumé.

On a étudié les possibilités d'inactivation de virus de dengue non modifié en passages de souris par du formol ou de la bile de bœuf. Le vaccin de dengue, ainsi préparé, a été appliqué expérimentalement à des souris blanches ainsi qu'à des volontaires humains.

Le vaccin formolé a été capable de protéger des souris contre des complications intracérébrales, provoquées par le virus de passage. Chez l'homme le vaccin inactivé par la bile de bœuf seul, mais non pas le vaccin formolé, était à même de produire, jusqu'à un certain point, une immunité protectrice contre une injection intradermale de virus humain virulent. Cependant un des quatre cas inoculés avec du vaccin à la bile de bœuf présentait des symptômes pouvant être interprétés comme une forme bénigne de dengue, probablement parce que dans le vaccin utilisé le virus n'a été atténué qu'incomplètement. Ceci amène à la conclusion qu'on doit attendre le résultat d'autres essais avant de procéder à l'application pratique du vaccin à la bile de bœuf.

Les problèmes virologiques et immunologiques en rapport avec cette question sont discutés dans ce travail.

Zusammenfassung.

Die Inaktivierung eines unveränderten Dengue-Virus-Stammes nach Mäusepassage durch Formalin und Ochsengalle wurde untersucht. Mit diesem Impfstoff wurde bei weißen Mäusen und beim Menschen experimentiert.

Formolvakzine konnte die geimpften Mäuse vor einer intracerebralen Übertragung des Virus schützen. Bei den Experimenten, für welche sich Menschen freiwillig zur Verfügung stellten, zeigte sich, daß wohl Ochsengalle, nicht aber Formolimpfstoff einigen Schutz bietet. In einem der vier Fälle, bei denen man mit Ochsengalle impfte, zeigten sich nachher Symptome, die auf eine mildere Form von Dengue hinwiesen. Dies hängt wahrscheinlich mit einer unvollständigen Abschwächung des Virus zusammen. Es ist deshalb angezeigt, die praktische Anwendung des Impfstoffes mit Ochsengalle weiter zu untersuchen.

Der Verfasser erörtert in der Folge noch die Probleme der Ansteckung und der Immunität des Dengue-Fiebers.