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RESEARCH ON DENGUE IN TISSUE CULTURE

V. Production of Anti-Dengue Antibodies in Tissue Cultures from Monkeys Inoculated with an Attenuated Type I Dengue Virus Strain*

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Nobuya FUJITA, Shigeru IZAKI, Katsuhiko ODA, Eiji KIMURA and Susumu HOTTA. *Research on Dengue in Tissue Culture. V. Production of Anti-Dengue Antibodies in Tissue Cultures from Monkeys Inoculated with an Attenuated Type 1 Dengue Virus Strain.* Kobe J. Med. Sci. 18, 143-152, September 1972——As a continuation of the previous studies (reported in this JOURNAL, 1966, 12, 199-205; Ibid, 1969, 15, 163-180), *in vitro* production of antibodies against dengue virus was investigated by means of tissue culture techniques. From monkeys inoculated with live dengue virus of human-attenuated strain (type 1, Mochizuki), tissues (spleen, lymph node, lung, liver, kidney and testis) were excised and cultivated by the plasma-embedded method. Culture fluids harvested at various times during cultivation were measured for neutralizing (NT) and hemagglutination-inhibiting (HI) titers against the original dengue virus. Sucrose density gradient centrifugation (Suc-DGC) and mercaptoethanol (ME) treatment were also performed to analyze the immunoglobulins.

The anti-dengue NT and HI activities were clearly demonstrated in culture fluids of spleen, lymph node, lung or liver. The same activities of testis culture fluids were very low, and those of kidney cultures varied between different specimens. Under the same conditions, tissue culture fluids from monkeys inoculated with the virus killed by formalin showed few NT or HI activities.

Of the HI-positive samples, the Suc-DGC profiles could be divided into two peaks, i. e., bottom fractions, corresponding to 19S globulins and ME-sensitive, and top fractions, corresponding to 7S globulins and ME-resistant. General tendencies were that the culture fluids harvested early after the beginning of cultivation possessed the two peaks, while those taken during later stages of cultivation had a single peak in the top fractions. The patterns were similar in the samples both from monkeys receiving a primary injection of virus and those receiving the booster injection. A tendency was noted, however, that the titers of the latter groups were higher than those of the former.

Virologic and immunologic significances of the data obtained are discussed, referring to previous works relating to *in vitro* production of antibodies.

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INTRODUCTION

Previous reports from our laboratory[4,7] described the development in humans and monkeys of anti-dengue neutralizing (NT) and hemagglutination-inhibiting (HI) antibodies, without showing any abnormal symptoms, after intracutaneous injection of an attenuated dengue virus strain (type 1, Mochizuki). The doses of virus sufficient to stimulate the antibody production were comparatively small. When the virus of the same dosage was killed by formalin, its capacity to induce antibody production greatly decreased. The data suggest that an asymptomatic infection took place in the subjects as a consequence of the virus inoculation, bringing about the production of antibodies. This paper will describe experiments which were designed to inquire into some aspects of the mechanism of antibody production by use of tissue cultures from virus-inoculated monkeys.

MATERIALS AND METHODS

Virus

Type 1 dengue Mochizuki strain virus cultivated serially in primary kidney cell cultures from Japanese monkeys (*Macaca fuscata*) was used. The infected culture fluid, centrifuged at 3,000 rpm for 10 minutes to remove cellular debris, was regarded as "live virus." "Formalinized virus" of the same strain was prepared by mixing the fluid with formalin (neutralized by the addition of magnesium carbonate) at a final concentration of 0.05% and holding it at 4°C for 2 weeks; the viral infectivity was completely destroyed by this treatment. The Mochizuki virus passed in mice intracerebrally was used as antigen for NT and HI tests.

Monkey

Healthy male Japanese monkeys (*Macaca fuscata*), 2 to 4-year-old, procured from the Japan Monkey Center, were employed. Prior to experiments it was ascertained that they had no anti-dengue antibodies or inhibitors in the serum.

Experimental procedure

(1) Each of the monkeys was intracutaneously injected with 0.4 ml of virus (0.1 ml each at 4 sites) at the flexor side of the arms.

(2) Four to seven days thereafter, the animals were anesthetized by an intraperitoneal injection of pentobarbital; and the tissues (lung, spleen, liver, kidney, lymph node and testis) were excised, which were then subjected to *in vitro* cultivation by the plasma-embedded method as indicated below.

(3) The tissues, minced into fragments of about 1 mm^3 in size, were placed in a tube (15×150 mm), the lower half of which had been wetted with heparinized chicken plasma. Usually 4 or 5 pieces were linearlly put at a distance of about 5 mm in each tube. Then a drop of chick embryo extract was introduced. After a firm clot was formed, 2 ml of culture medium (consisting of 0.5% lactalbumin hydrolysate and 10% inactivated bovine serum in Hanks' salt solution supplemented with 100 u/ml penicillin and 100γ/ml streptomycin) was added. It was ascertained that the

medium had no anti-dengue antibodies or inhibitors. In every series of experiments, at least 10 tubes were prepared for each kind of the tissues.

(4) Two to 14 days after the beginning of incubation at 37°C in the stationary state, portions of the culture fluid were removed from each tube and pooled with others from similarly incubated tubes. After being inactivated by heating at 56°C for 30 minutes, the fluid was tested for anti-dengue NT or HI activities.

(5) The NT titers were determined by the mouse-intracerebral method as described previously.[7] For the HI tests, the fluids were pre-treated by the procedures as previously described,[6] and the microtiter techniques (Takatsy-Sever)[13, 6] were applied; specifics for the preparation of antigen were the same as stated previously.[6] Using the selected positive samples, sucrose density gradient centrifugation (Suc-DGC), together with 2-mercatoethanol (ME) treatment, was performed to analyze the antibodies; the methods were the same as described in the previous paper.[4]

Fig. 1 shows a scheme of the whole procedure.

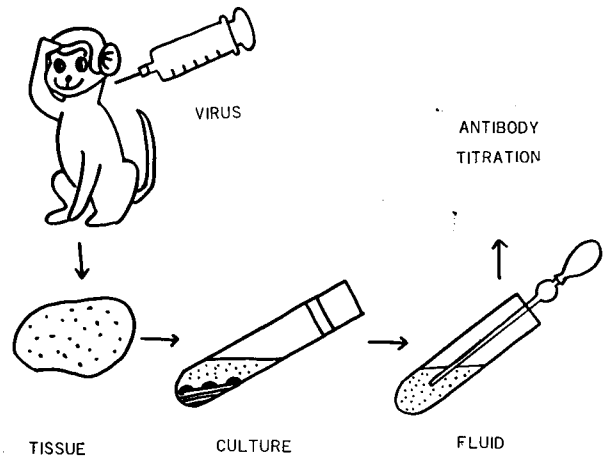


Fig. 1 Schematical illustration of experimental procedures.

RESULTS

Preliminary results from separate series of experiments are summarized in Table 1.

It is clear that some of the culture fluids from monkeys inoculated with the live virus showed anti-dengue NT activities of significant degrees. Practically no difference was noted between the titers of groups inoculated with undiluted virus and those with 100-fold diluted virus. However, the similar specimens from monkeys inoculated with the formalin-killed virus showed much weaker activities, almost to the point of none.

In subsequent experiments, the culture fluids were taken for test at regular intervals during cultivation. Typical examples of the results obtained are illustrated in Fig. 2.

Table 1 Anti-dengue neutralizing activity of tissue culture fluids from monkeys inoculated with human-attenuated dengue virus (type 1 Mochizuki strain)

Monkey no.	1	2	3	4	5	6
Virus inoculated	Live, undiluted[a]		Live, 100-fold diluted		Formalinized	
Duration, in days, from virus inoculation to excision of tissue	4	7	4	4	4	7
Tissue culture						
Spleen	1.5[b] (5)[c]	3.0 (14)	1.0 (14)	1.0 (8)	0.5 (14)	0 (8)
Lymph node	0.25 (5)	2.0 (14)	1.65 (8)	0.75 (8)	0.5 (14)	0 (8)
Lung	0.5 (8)	3.0 (14)	1.25 (8)	0.75 (8)	1.0 (8)	0 (8)
Kidney	1.38 (14)	0.25 (14)	1.25 (8)	1.25 (8)	0.25 (8)	0.25 (8)
Liver	1.5 (5)	1.75 (14)	1.62 (8)	1.0 (8)	0.5 (14)	0 (8)
Testis	0.5 (5)	0 (14)	0.62 (8)	0.75 (8)	0.25 (8)	0 (8)

a: Infective titers were in the range of $10^{4.75}$–$10^{6.0}$ in mouse-intracerebral LD_{50} per 0.02 ml.

b: Netralization index, as measured by the mouse-intracerebral method. Culture fluid and dilutions of virus (mouse-passaged) were mixed in equal volume; after the mixtures were held at 37°C for 1 hour, 0.02 ml thereof was injected into mice intracerebrally; mixtures of the same virus and uninfected culture fluid were used as a control; differences of LD_{50}'s between the test fluid and control fluid groups were regarded as the indices.

c: Parenthesis indicates duration, in days, of cultivation at 37°C. Every 4 or 5 days, the culture medium was changed at 90% level

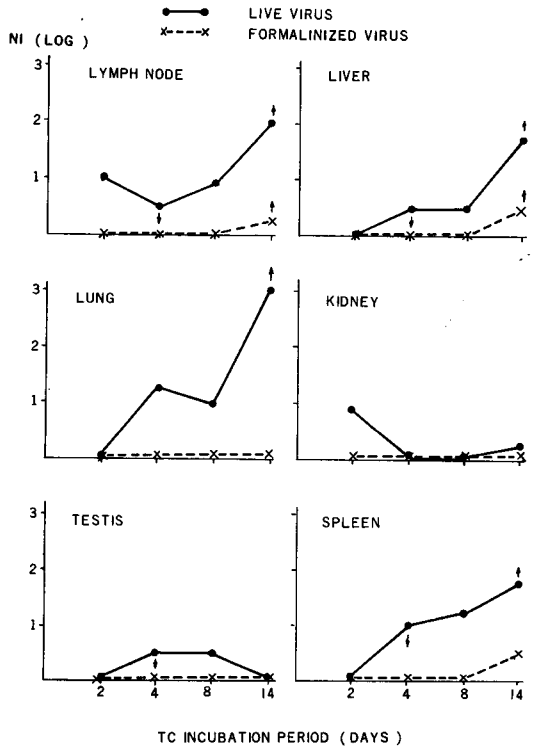


Fig. 2 Neutralizing activities of tissue culture fluids from monkeys inoculated with live or formalinized dengue virus (human-attenuated type 1 Mochizuki strain). Ordinate indicates anti-dengue neutralization indices (in log), and abscissa indicates period of tissue culture incubation in days. (Reproduced from Hotta, S., Dengue and Related Hemorrhagic Diseases, 1969, Warren H. Green, Inc., St. Louis, U. S. A.)

It is again shown that culture fluids from monkeys inoculated with the live virus had significant NT activities which, as a general tendency, increased in titer in the course of incubation. The NT titers of fluids from spleen, lung, lymph node or liver cultures were comparatively high, while those of testis cultures were low. In the kidney cultures, the appearance of NT activity in fluid varied from one monkey to another; the samples from some monkeys exhibited fairly high activities, while those from others did not. Distinct from the data of "live virus" groups, similar specimens from the "formalinized virus" groups showed practically no activity, except for a few cases which showed clear but much less titers. The same tendencies were noted in repeated experiments similarly performed.

Using the samples as indicated in Table 2, Suc-DGC and ME-treatment were carried out. Examples of the data are shown in Figs. 3 and 4.

Table 2 Tissue culture fluids used for sucrose density gradient analysis and mercaptoethanol treatment

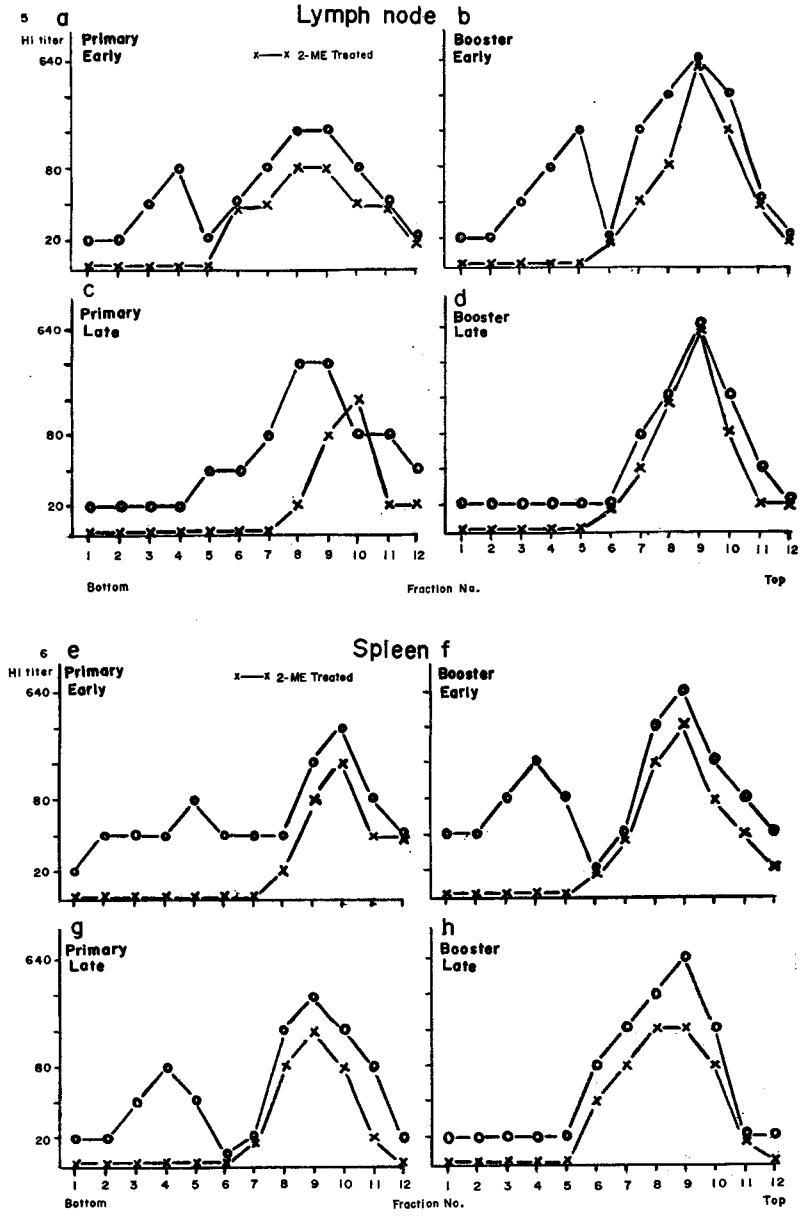
Tissue culture	Fluid	Group[1]	
		Primary injection[2]	Booster injection[3]
Lymph node	"Early"	a (5)[4]	b (5)
	"Late"	c (21)	d (21)
Spleen	"Early"	e (5)	f (5)
	"Late"	g (21)	h (21)

1. As to the designations (a)-(h), refer to Figs. 3 and 4.
2. Four days after a primary injection, the tissues were excised for cultivaton.
3. Two shots of virus were given at a 2-week-interval; and 4 days after the second injecion, the tissues were taken.
4. Period, in days, from beginning of cultivation to harvest of culture fluid. Every four or five days during the cultivation, the culture medium was changed at 90% level.

(1) The Suc-DGC profiles expressed by HI titers exhibited two peaks, i. e., bottom and top fractions. The former, corresponding to the 19S immunoglobulins, was ME-sensitive, and the latter, corresponding to the 7S immunoglobulins, ME-resistant.

(2) General tendencies were noted which showed that the titers of the booster injection groups were higher than those of the primary injection groups, and that the titers of the top fractions were higher than those of the bottom fractions.

(3) The "early fluids" (taken early after the beginning of cultivation), both from the primary injection and booster injection groups, generally had two peaks. However, the majority of the "late fluids" (taken in later stages of cultivation) possessed only one peak in the top fractions. In other words, the anti-dengue HI activities of culture fluids taken early in cultivation were present both in the bottom and top fractions, whereas the same activities of fludis taken later were mainly found in the top fractions.



Figs. 3 and 4 Results of sucrose density gradient centrifugation and mercaptoethanol treatment of tissue culture fluids from monkeys inoculated with live dengue virus (human-attenuated Mochizuki strain).

Ordinate indicates anti-dengue HI titers (reciprocals of highest dilution of serum showing positive reaction) and abscissa indicates number of fractions from a centrifuge tube.

As to the designations (a)-(h), refer to Table 2.

DISCUSSION

The problem of how the antibody is produced has been investigated from various angles. One of the approaches to solving it is utilization of tissue culture techniques. Two methods have been adopted for this purpose. One is to put antigen in contact with cells already cultured *in vitro*. The first report of this category is perhaps that by Carrel and Ingebrigtsen[3] who claimed to demonstrate anti-sheep hemolysin in guinea pig spleen and lymph node tissue cultures which had been maintained *in vitro* and then were added with sheep erythrocytes. Other investigators, however, e. g., Kucynski et al,[9] failed to obtain positive results in their experiments performed along similar lines. More recent studies utilizing newer techniques, on the other hand, presented some evidences for a primary antigenic stimulation in cell cultures from non-immune animals (Refer to a review article by Bussard[2]).

The second method consists of taking tissues or cells from animals previously administered with antigen and then cultivating them *in vitro*. Lüdke[10] demonstrated anti-dysentery or anti-typhoid agglutinins in extracts of spleen and bone marrow tissues from rabbits or guinea pigs which had previously been injected with the bacilli. Later works of the similar type, particularly those by Kimura and his colleagues,[8] have indicated that this method is adequate to bring about appearance of specific antibodies in culture fluids cleary and regularly (As to the historical and critical comments, refer to review articles[5, 11, 14]).

The present study was conducted applying the second method. Anti-dengue NT and HI activities of significant titers appeared in fluids of tissue cultures from Japanese monkeys which had previously been inoculated with live dengue virus. The comparatively high titers were shown in the cultures of spleen, lymph node, lung or liver, while the titers in testis cultures were low. The same activities revealed in the kidney cultures varied between different specimens; the titers of some cases were relatively high and those of others low. Few activities of the same nature were found in similar cultures from monkeys inoculated with the virus killed by formalin.

The Suc-DGC and ME treatment studies indicated that the activities could be divided into two parts, i. e., bottom fractions, corresponding to 19S globulins and ME-sensitive, and top fractions, corresponding to 7S globulins and ME-resistant. Fluids obtained early after the beginning of cultivation had the two parts, while those taken during later stages of cultivation showed usually a single peak of activity in the top fractions. The similar tendencies were noted in the samples both from monkeys receiving a primary injection of virus and those receiving the booster injection, although the tiers of the latter groups were generally higher than those of the former.

The data indicate that so-called 19S and 7S immunoglobulins against dengue were produced by the tissues which were taken from virus-inoculated monkeys and then cultivated *in vitro*. It was noted that the virus of the living state was more effective in the antigenic stimulation than was the formalinized virus of the same dosage, so far as under the conditions studied.

No conclusive statement can be made in the present study as to the problem of what kind(s) of cells are involved in the production of antibodies. Since, however, the positive results were obtained, in general, with the cultures of spleen, lymph node, lung and liver, it is very likely that cells composing these tissues, probably of the reticulo-endothelial system, are principally responsible for the antibody production. Major components of the testis cultures are usually fibroblasts which are believed to have little function in producing antibodies. The positive data of kindney cultures in the present experiments may be worth discussion. Previous works, e. g., those by Sakurai[12] or by Askonas and Humphrey,[1] showed negative results concerning antibody production by kidney tissue cultures. Although no reason(s) for this discrepancy are clear, one point to be stated in this connection is that the combination of dengue virus and monkey is a natural system of infection. Our previous experiments (unpublished) revealed formation of mononuclear cell infiltration foci, though relatively small in extent, in the kidney of monkeys inoculated with the live Mochizuki virus (attenuated strain). It is possible to assume that cells composing such infiltration foci produce antibodies when cultivated in tissue cultures. This also may explain the varying data on the antibodies detectable in kidney cultures from different monkeys.

The *in vitro* production of antibodies is probably a basis of the appearance of the same antibodies in the serum of animals after inoculation of antigens. In the case of monkey tissue cultures versus dengue virus, the fact that this combination is a natural system of infection must be additionally taken into consideration.

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