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SYNOPSIS

Follow-up serological studies of dengue were conducted with sera from residents of Nagasaki who had suffered from the disease during the large dengue epidemics in 1942-44.

The specific anti-dengue antibodies were significantly retained in some of the serum samples obtained at least 32-36 years after what was most probably a single attack of dengue. The antibodies, distinguished from anti-Japanese encephalitis antibodies, appeared in the IgG fractions. These data gave an supporting evidence that the Nagasaki epidemics in 1942-44 were due to a dengue-1 virus. The epidemiological significance of these results are discussed.

INTRODUCTION

In 1942 an epidemic of dengue (DEN) occurred in Nagasaki, Japan, and a few other cities in Japan. The disease recurred each year until 1944. Since then, however, the disease has disappeared completely, and no dengue case, either typical or suspected, has been recognized.

In the previous paper, we reported that sera taken from residents of Nagasaki revealed clearly specific anti-dengue (type 1) antibodies 20 years after the first occurrence of the epidemics.[3)]

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Similar aspects were described by other investigators using sera from residents in Florida, U.S.A.,[2,9] in Greece[5,6,11] and in Panama.[7,8] The long persistence of anti-dengue antibodies in humans is therefore apparent.

In the present paper, we report data of our further study in which the sera taken 32 to 34 years after the 1942-44 epidemics in Nagasaki were examined for their anti-dengue antibodies. Additional tests were carried out as an experimental basis of the epidemiological results obtained.

MATERIALS AND METHODS

Test Serum.

Serum samples were collected from 9 residents of Nagasaki in November 1976 and October 1978 and stored at -80°C until ready for use. All the subjects had suffered from dengue during the 1942-44 Nagasaki epidemics and, residing there since then, had no experience of entering dengue-endemic areas. Because of their exposure to the atomic bomb attack in 1945, they have received regular medical examinations by the Radiation Effects Research Foundation of Japan. According to their records, the possibility of their contracting flavivirus infections, excepting Japanese encephalitis infection (most probably of inapparent nature), was practically nil.

Serological Tests.

Neutralization (NT), hemagglutination-inhibition (HI) and complement-fixation (CF) tests were performed.

Antigens: The following antigens were prepared from infected suckling mouse brains: Dengue type 1 (DEN-1), Mochizuki and Hawaiian strains; DEN-2, Trinidad 1751 strain; DEN-3, H-87 strain; DEN-4, H-241 strain; and Japanese encephalitis (JE), G1 and JaGAr01 strains.

NT test: The serum was inactivated by heating at 56°C for 30 minutes just prior to testing. After two-fold dilutions of serum and a fixed amount of virus were mixed, the serum-virus mixtures were incubated at 37°C for 1 hour and held in an ice bath for 20 minutes. Then they were inoculated into BHK-21 cells which had been cultivated either in 3 oz plaque bottles or in wells of tissue culture microplate (Linbro's TISSUE CULTURE multi-well plate with 24 flat bottom wells, 1.7 x 1.6 cm in size). The

infected cells were cultivated at 37°C under an overlay medium consisting of 1% methylcellulose (MC) and 2% heat-inactivated calf serum in Eagle's minimum essential medium (MEM). Seven to 9 days later, the cells were washed with Hanks' balanced salt solution (BSS) and fixed and stained with 1% crystal violet solution in 20% alcohol. Fifty percent plaque reduction neutralization (PRN_{50}) titers were calculated by the Behrens-Kärber method.

HI and CF tests: Antigens were prepared by the sucrose-acetone method of Clarke and Casals.[1)] In the case of HI, the sera, without being heat-inactivated, were treated with acetone and goose erythrocytes. For the CF tests the heat-inactivated sera were used. Microtiter modification methods were adopted.

Immunoglobulin Analysis.

The sera treated with acetone and goose erythrocytes were centrifuged at 100,000 g for 16 hours in a sucrose density gradient (10-40% w/v) using Beckman SW50.1 rotors. The fractions obtained were measured for the HI titers.

RESULTS

Using the sera from 9 individuals, this study measured the NT, HI and CF antibodies against all serotypes of DEN and JE viruses. The results are shown in Table 1.

Anti-DEN-1 NT antibodies were detected in 7 out of the 9 sera, among which two samples, Nos. 3 and 4 in Table 1, showed comparatively high titers against DEN-1, i.e., 155 and 978, respectively. Anti-DEN-2 NT antibodies of low levels were also demonstrated in 4 sera. No NT antibodies against DEN-3 and DEN-4 were found in any of the 9 sera. Anti-JE NT antibodies were detected in 8 sera. The anti-DEN and anti-JE antibodies were not parallel with each other.

The 6 sera possessed HI antibodies against DEN-1 virus, whose titers against the Mochizuki and Hawaiian strains were of the same level in all the cases. The 4 sera reacted in HI against 2 or more serotypes of DEN viruses. One sample, No. 7, had no detectable antibodies. In general, the HI antibodies showed tendencies similar to the NT antibodies, although the former were more broadly reactive than the latter.

The CF antibodies of low levels were demonstrated in some of

Table 1 Anti-DEN and JE antibodies in serum from residents of Nagasaki.

Serum no.	Age	Sex	PRN_{50} against					HIU/0.025 ml against						CFU/0.025 ml against				
			D1 Mochi	D2 Tr	D3 H87	D4 H241	JE G1	D1 Mochi	D1 Haw	D2 Tr	D3 H87	D4 H241	JE JaGAr	D1 Mochi	D2 Tr	D3 H87	D4 H241	JE JaGAr
1	70	F	62	-	-	-	116	-	-	-	-	-	40	-	-	-	-	-
2	71	M	43	-	-	-	61	-	-	-	-	-	-	-	-	-	-	-
3	64	M	155	29	-	-	12	60	40	-	-	-	-	-	-	-	-	-
4	51	M	978	25	-	-	22	160	160	20	20	-	30	32	8	8	6	4
5	50	M	74	-	-	-	378	20	20	-	-	-	40	-	-	-	-	-
6	74	M	29	41	-	-	720	80	80	80	30	80	160	-	-	4	-	4
7	69	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	48	F	-	18	-	-	52	20	20	20	20	40	80	-	-	-	8	-
9	45	M	46	-	-	-	574	20	20	20	-	-	80	-	-	-	-	4

D1 Mochi, DEN-1 Mochizuki strain.
D1 Haw, DEN-1 Hawaiian strain.
D2 Tr, DEN-2 Trinidad 1751 strain.
D3 H87, DEN-3 H-87 strain.
D4 H241, DEN-4 H-241 strain.
JE G1, JE G1 strain.
JE JaGAr, JE JaGAr01 strain.
-, < 10 PRN_{50}, < 20 HIU/0.025 ml or < 4 CFU/0.025 ml.
The HI and CF tests were conducted in duplicate employing 8 HA units of the antigen for HI and 2 exact units of complement for CF.

the samples. Serum No. 4 reacted in CF against all serotypes of DEN and JE as well, showing the highest titers against DEN-1 NT and HI.

Immunoglobulins were separated from the sera which were positive in HI against DEN-1 and DEN-2. Table 2 shows the data obtained.

Table 2 HI reactivities of immunoglobulins separated from test serum.

Serum no.	HIU/0.025 ml against					
	D1-Mochi			D2-Tr		
	Whole serum	IgM	IgG	Whole serum	IgM	IgG
3	60	-	40	-	-	-
4	160	-	160	30	-	20
5	20	-	-	-	nt[a)]	nt
6	80	-	40	80	-	40
8	20	-	20	20	-	20

a) Not tested.
Other legends are the same as those in Table 1.

It was clear that the antibodies were present entirely in the IgG fractions.

To investigate the specificity of the detected anti-DEN-1 NT antibodies, we carried out cross-NT tests between DEN-1 and JE. Typical examples of the results are shown in Table 3.

The homologous reactions were unequivocally observed at high levels, but no overlapping was revealed in the heterologous tests. The anti-DEN-1 and anti-JE NT antibodies were clearly distinguishable from each other, at least under the conditions studied.

Table 3 Cross-NT tests between DEN-1 and JE viruses.

Antigen[a]	Antibody to[b]	
	DEN-1	JE
DEN-1	1430[c]	< 10
	1150[d]	*< 10*
JE	< 10	13300
	< 10	*12000*

a) Antigen: DEN-1, mouse-passaged Mochizuki strain; JE, mouse-passaged G1 strain.
b) Antibody: anti-DEN-1, immune mouse ascitic fluid, kindly provided by Dr. N. Karabatsos and Dr. R. E. Shope, Yale University; anti-JE, hyperimmune rabbit serum.
c) PRN_{50} titers obtained using 3 oz plaque bottles.
d) Figure expressed in italic, obtained by the semi-micro method using Linbro's TISSUE CULTURE multi-well plates.

DISCUSSION

The anti-DEN NT, HI and CF antibodies were found in the sera collected in 1976-78 from the residents in Nagasaki who suffered from DEN during the Nagasaki epidemics in 1942-44. The anti-DEN-1 antibodies were particularly positive. This finding confirmed that the antibodies persisted for 32-36 years after the previous dengue infection. The anti-DEN-2 NT antibodies were also detected, but their levels were very low. While the demonstrated anti-DEN-2 antibodies were presumably the result of cross-reaction by other DEN serotypes, especially DEN-1, problems of whether or not the DEN-2 virus was involved in the Nagasaki epidemics deserve further consideration. No anti-DEN-3 and DEN-4 NT antibodies were detected in any of the sera, at least within the scope of our present survey.

The specificity of the observed DEN-1 antibodies is worth some discussion. It is quite possible that many residents of Japan possess anti-JE antibodies probably induced by repeated

inapparent infection(s) or by JE vaccination. While this may be the case of the present study, the anti-DEN-1 and anti-JE antibodies detected did not coincide with eath other (Table 1). Our findings strongly suggest, therefore, that the anti-DEN-1 NT antibodies observed in the present survey were specific. It should be pointed out that no flavivirus infection, excepting JE, has been recognized in Japan; hence the possibility that the persons whose sera were examined in the present study had contracted any other flaviviruses can be logically excluded. Moreover, in the cross-NT tests using immune mouse ascitic fluid and hyperimmune rabbit serum, the anti-DEN-1 and JE NT antibodies could be clearly distinguished from each other. Similar aspects were already reported by Smithburn[10] and Hotta et al.[3] The cross reactions between DEN and JE NT antibodies appear to be negligible. In the light of the above statements, it was concluded that the specific anti-DEN antibodies have persisted for more than 30 years after a single infection of dengue. It should be added in this connection that, since the DEN-1 Mochizuki strain virus employed in this study was isolated from a dengue patient at Nagasaki in 1943,[4] it is evident that the dengue epidemic of Nagasaki was due to DEN-1 virus.

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