

LYMPHOCYTOTOXIC ANTIBODY IN SERUM FROM PATIENTS
WITH DENGUE HEMORRHAGIC FEVER (DHF)

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OBJECTIVE : To identify lymphocytotoxic activity in the sera of Thai children with dengue hemorrhagic fever.

BACKGROUND : Lymphocytotoxic antibodies (ALA) have been reported for a variety of natural, pathological, and disease states (1, 2, 3, 4). Earlier studies were concerned primarily with the appearance of anti-lymphocytotoxic antibodies directed against specific allogenic transplantation antigens (5, 6).

However, recent interest has focused on spontaneously induced autocytotoxic antibodies and their relationship to dysfunctions seen in several disease states (7, 4). Some of these are warm antibodies, but the majority react optimally, or only, in the cold (8) and virtually nothing is known about the effect of these ALA on peripheral blood cells *in vivo*.

Our laboratory previously reported that Thai children with dengue hemorrhagic fever (DHF) showed a major shift within the component cell populations of the immune system (9). The major changes noted in the lymphocyte subpopulations were a decreased percentage and number of T lymphocytes with an increased percentage and number of non-T, non-B, non-Fc receptor bearing cells during the acute stage of illness.

Various causes for the alteration in lymphocyte subpopulation during viral infections have been suggested by several authors (10, 11). Recently, Boonpucknavig (12) used indirect immunofluorescence to detect anti-T-lymphocyte antibodies in sera from patients with dengue hemorrhagic fever. However, the study did not determine the incidence of anti-T-lymphocyte antibodies in sera from patients with DHF or whether these antibodies were cytotoxic for autologous lymphocytes.

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The present study was undertaken to determine if sera from Thai children with DHF was cytotoxic for autologous peripheral blood cells and if such activity could be responsible for the alteration of T lymphocyte numbers during DHF illness.

: Heparinized peripheral blood samples were drawn from Thai children examined at the Bangkok Children's Hospital, Bangkok, Thailand, after they were clinically diagnosed as having acute dengue hemorrhagic fever (DHF) and after informed consent was obtained from the Children's parents. All cases were grouped into those with and without dengue shock syndrome (DDS) as reported elsewhere (13). Serological confirmation and classification of primary or secondary illness was performed as previously described (9).

Convalescent blood samples were subsequently collected from some confirmed DHF cases 15 and 30 days after the initial blood samples were drawn. Peripheral blood was drawn from a small number of cases 5 to 10 months later.

Control peripheral blood mononuclear cells used in the lymphocytotoxicity screening assays were obtained from healthy Thai adults while mononuclear cells used later for determining the specificity of the lymphocytotoxicity for lymphocyte subpopulations were obtained from healthy Caucasian adult volunteers.

Isolation of Peripheral Blood Lymphocytes and Lymphocyte Subpopulations :

Peripheral blood lymphocytes were prepared from venous blood centrifuged through Ficoll-Hypaque (14) and then washed three times with Hanks Balanced Salt Solution (HBSS), then resuspended in RPMI 1640 supplemented with L-glutamine, HEPES, and 10% heat inactivated FCS. Initially each serum was screened for the presence or absence of lymphocytotoxicity against autologous or allogenic DHF patient, and control lymphocytes.

Aliquots of some patient serum were frozen, packed in dry ice and shipped to Washington Univ., St. Louis, Mo., where the sera were tested for the presence of lymphocytotoxicity against a panel of lymphocytes from 15 normal Caucasian adults of varying HLA-phenotype. Unused serum was stored at -70°C until used.

Subsequently, mononuclear cells from a small number of donors were used to determine if the serum lymphocytotoxicity was specific for lymphocyte subpopulations. Mononuclear cells were isolated from a unit of peripheral blood from 5 of the initial healthy Caucasian volunteers by Ficoll-Hypaque centrifugation. The cells were further separated into subpopulations as previously described (15).

Briefly, the isolated cells were passed over a Sephadex G-10 column to remove adherent cells. The nonadherent macrophage deplete cells were separated into T, B, and Null cells in a two stage process. First, the cells were poured over a Sephadex G-200 anti-human $(\text{F}(\text{ab}')_2)$ immunoabsorbent column. Then the nonadherent T plus Null cells were further fractionated using on overnight incubation with SRBCs followed by Ficoll-Hypaque density centrifugation. The adherent cells (B cells) were eluted from the column with medium containing 1% human immunoglobulin, incubated overnight with SRBC, and centrifuged over Ficoll-Hypaque.

Surface characteristics determinations of the enriched cell populations were performed to quantitate the percentage of SIg⁺ cells, E-rosette⁺ cells and esterase⁺ cells as previously described (15). All populations were highly enriched and viability of the final preparation was greater than 95%, as assessed using the trypan blue dye exclusion procedure.

Microcytotoxicity Assays : The initial screening of sera for the presence or absence of lymphocytotoxicity was performed using the previously described modification (4) of the method of Terasaki and McClelland (16). Briefly, assays were done in duplicate in 96 well flat bottom microtiter trays. 0.1 ml of target cell suspension (3×10^6 lymphs/ml) was added to each well containing 0.1 ml test serum, then incubated at either 4°C or 37°C for 30 minutes. Then 0.1 ml of fresh rabbit serum was added as a source of complement and the microplates were incubated at 15°C or 37°C for an additional 4 hours. In a small number of cases the incubations were performed at 4°C or 30°C. Then the contents of each well was resuspended and one drop of the cell suspension was mixed with one drop of 2% eosin Y on a microscope slide. After allowing 2 minutes for dye uptake a glass cover slip was put over the cells and target cell death was assessed using a phase contrast microscope.

To determine complement dependency, test sera were run in parallel, adding complement to one group and deleting the complement from the other. To maintain the volume, 0.1 ml of media was added to the plate not receiving complement. When complement was not added, all test sera were negative for lymphocytotoxicity. In a small number of assays patients' plasma was also tested for the presence or absence of lymphocytotoxicity.

Serum with high lymphocytotoxicity from malarious patients or patients with systemic lupus erythematosus served as positive controls while serum from healthy Thai volunteers served as negative controls.

The microcytotoxicity assays were modified slightly when serum was tested for lymphocytotoxicity against enriched T-cell, B-cell, or Null cell targets. Serum was distributed on microtiter trays which had been covered with mineral oil, and then .001 ml serum was added to each well and the trays were stored at -70°C until needed. The assays were performed by putting .001 ml of target cell suspension (2×10^6 lymphs/ml) into each well containing test serum. The target cell-serum mixture was incubated 1 hour then .050 ml rabbit complement (Pel-Freeze) was added to each well and the mixture further incubated for 3 hours. .003 ml of 5% aqueous eosin was then added to each well and 2 minutes later .010 ml of formalin was added to the wells and the tray covered with a 50 x 75 mm microscope slide. Target cell death was determined using a phase contrast microscope.

To study thermal characteristics of the cytotoxic antibodies, the tests were done at two temperatures. The initial cell/serum incubation was done at either 4°C or 37°C, followed by the 3 hour complement incubation at either 15°C or 37°C respectively.

Positive sera controls included sera from SLE and malarious patients while negative control sera included sera from patients with primary biliary cirrhosis or healthy volunteers.

Scoring of sera lymphocytotoxicity against target cells was done using the following scale :

- 1 - negative 0-10% cell dead (same as negative control)
- 2 - doubtful negative 11-20% cells dead
- 4 - doubtful positive 21-50% cells dead
- 6 - positive 51-80% cells dead
- 8 - strong positive 81-100% cells dead

Serum Fractionation : Serum with high lymphocytotoxicity was fractionated using sucrose density centrifugation by layering 0.3 ml of serum over approximately 6.9 ml of a 10% to 40% linear sucrose gradient (Kunkell, G.H., 1960). After centrifugation twelve equal fractions, 0.6 ml per fraction, were collected after carefully puncturing the bottom of the centrifuge tubes. Immunodiffusion plates were used to determine which fractions contained IgG or IgM. Fractions containing only IgG or IgM were pooled prior to testing for lymphocytotoxicity. Preliminary experiments were performed with sera containing lymphocytotoxic antibody to ensure that the sucrose concentration in the various fractions did not interfere with the ability of patient sera to kill lymphocytes. Each fraction was tested against normal unfractionated mononuclear cells at 4°C using the microcytotoxicity assay to determine which fractions contained lymphocytotoxicity.

RESULTS : Sera from patients with confirmed dengue hemorrhagic fever demonstrated optimal cytotoxic activity against peripheral blood mononuclear cells at temperatures below 37°C.

The levels of cytotoxic activity against target indicator cells from healthy Thai adults are shown in Table 1. During acute illness serum cytotoxic activity was only demonstrated when used in assays performed at 15°C or 4°C. No significant cytotoxic activity was found in assays done at 37°C. The small amount of serum obtained from individual patients prevented us from testing the serum at more than two different temperatures.

When we compared the cytotoxic activity of serum and plasma from the same individuals no activity could be demonstrated in plasma (Figure 1). Further tests will be needed to determine the reason for the absence of cytotoxic activity in plasma and to determine if heparin has any effect on anti-lymphocytotoxic activity.

However, if individual patient serum was examined for the presence of anti-lymphocytotoxic antibodies in assays done at 15°C, anti-lymphocytotoxic killing activity ranged from a low level 4.5% to a high level of 58.5%. As can be seen from the comparison with control sera in Figure 2 the average level of activity for the 90 DHF patients is 18.7% while the average level of activity for the control sera is 6.0. If an arbitrary value greater than 10% killing of target cells is used to indicate positive anti-lymphocytotoxic activity, 59/90 DHF samples are positive while none of the controls are positive.

When allogenic target indicator cells are used in the serum microcytotoxicity assay, no significant difference in the level of serum cytotoxic activity against mononuclear cells from other DHF patients or healthy Thai adults is seen in Table 2.

18/25 sera obtained during the convalescent period (30 days post admission) of illness from patients whose serum demonstrated cytotoxic activity during the acute stage of illness had a lower level of cytotoxic activity (Table 3) than the sera obtained on the day of presentation to the hospital. Since none of the sera were heat inactivated prior to storage at -20°C we ruled out the possibility of our processing and storage procedure being responsible for any fluctuation in the level of cytotoxic activity in Table 4.

Because it is not unusual for the temperature of a patient with DHF to drop to $30-31^{\circ}\text{C}$ at some time during the course of illness we looked for anti-lymphocytotoxic activity in unfractionated sera used in assay done at the $30-31^{\circ}\text{C}$ temperature. However we have not been able to demonstrate any activity at $30-31^{\circ}\text{C}$ in unfractionated sera with high levels of cytotoxic activity at 15°C .

When sera were diluted and examined for cytotoxic activity there was no evidence of a prozone phenomenon being responsible for some sera demonstrating low or negative cytotoxic activity at low dilutions. Table 5 shows the effect that freezing thawing and/or dilution of sera with high lymphocytotoxic activity has on serum lymphocytotoxicity.

When 13 DHF sera that were known to have high lymphocytotoxic activity were tested against enriched T-cell, B-cell or Null cell populations 23% demonstrated no activity, 31% showed activity against both T-cells, and B-cells at 4°C , 8% showed activity against only T-cells at 4°C , 15% showed activity against B-cells at 4°C , 23% showed activity against B-cells at both 4°C and 37°C . No reactivity was seen when enriched null cell populations were used as target indicator cells.

In preliminary sucrose gradient serum fractionation studies lymphocytotoxicity is found in the fractions known to contain IgM. Interestingly, the IgM fractions obtained from whole sera which previously demonstrated no lymphocytotoxicity at 37°C , is cytotoxic to unfractionated mononuclear cells when assayed at both 4°C and 37°C . Additional experiments are underway to determine if IgG fractions contain blocking factors that interfere with IgM lymphocytotoxicity at 37°C . We are also trying to determine the target cell specificity of the specific sucrose gradient fractions at both 4°C and 37°C .

Lastly we are analysing our data to determine if any correlation exists between lymphocytotoxic activity and age, sex, day of illness, grade of illness, HI titer, differential blood data, or patients total WBC count.

Table 1. Comparison of DHF Sera Cytotoxicity at Different Temperatures

<u>Patient #</u>	<u>4°</u>	<u>15°</u>	<u>37°</u>
62	-	23.5	8.5
66	-	4.5	4.5
69	-	6.0	4.0
70	-	9.0	7.0
71	-	13.0	6.0
72	-	42.0	7.0
75	-	24.0	7.0
76	-	27.5	6.5
78	-	8.0	7.0
79	-	12.5	5.5
80	-	10.0	8.5
81	-	51.0	8.0
82	-	35.5	7.5
83	-	7.0	6.0
208	21.0	23.5	-
209	13.5	14.5	-
211	6.0	8.0	-
212	11.0	7.0	-
216	17.0	24.0	-

Table 2. Cytotoxic Activity of DHF Serum Against Different Target Cells

Serum**	Mononuclear Target Cells*		Normal
	Autologous	Allogeneic	
1	-	7.0	6.5
2	28.0	-	35.5
3	18.5	27.0	20.5
4	6.5	10.5	11.5
5	5.5	-	6.5
6	-	19.5	24.0
7	-	11.0	14.0
8	-	14.0	13.5
9	-	6.5	7.0
10	6.5	-	7.5
11	-	6.0	10.0
12	-	23.5	27.0
13	6.5	6.0	5.5
14	9.5	12.0	14.5
15	-	10.0	13.5
16	-	10.5	10.0
17	8.5	-	12.0
18	-	11.0	16.0
19	-	7.5	10.0
20	7.0	-	10.5
21	5.0	-	8.0
22	7.0	9.0	7.5
23	34.0	30.5	38.0
24	-	24.5	21.5
25	-	12.0	14.5
26	8.5	-	14.5
27	14.5	-	11.0

* Assay tested at 15°C

** All sera obtained during acute stage of DHF illness

Table 3. Acute vs Convalescent DHF Sera Cytotoxic Activity

	<u>0</u>	<u>15 day</u>	<u>30 day</u>	<u>Control</u>
1	13.5*	6.5	7.0	5.0
2	44.0	24.0	31.0	7.5
3	7.5	7.0	7.5	7.5
4	16.5	7.0	23.0	5.5
5	22.5	5.5	15.0	5.5
6	5.5	6.0	6.0	5.5
7	9.5	6.0	6.5	6.5
8	13.0	13.5	14.0	6.5
9	22.0	23.0	20.5	6.5
10	17.0	8.0	7.5	5.5
11	19.0	15.0	6.0	6.0
12	28.0	27.5	19.0	4.5
13	24.5	5.5	9.5	7.5
14	30.5	49.0	55.5	7.5
15	16.5	16.0	13.5	7.5
16	11.0	6.5	6.0	6.0
17	32.5	24.0	8.0	6.0
18	32.0	22.0	8.5	6.0
19	8.0	16.0	11.5	6.0
20	37.0	22.0	6.0	6.5
21	27.0	22.0	17.5	6.5
22	11.5	16.0	9.5	6.5
23	44.0	50.0	32.5	6.5
24	28.0	30.0	29.5	6.5
25	27.5	21.5	16.0	6.5
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S.D.	11.036	12.46	11.66	0.804
Mean	21.92	17.82	15.46	6.3
Var.	116.91	149.118	130.598	0.62

* Test at 15°C. All sera was stored without heat-inactivation. Heat inactivation of serum was done at 56°C for 30 min. prior to use in microcytotoxicity assays.

Table 4. Effect of Processing and Storage on Cytotoxic Activity of DHF Sera with Control Subtracted

	<u>HI then freeze thaw⁺</u>			<u>Freeze thaw then HI⁺⁺</u>	
1	15.0	8.5		16.0	15.0
2	43.5	36.5		21.5	23.5
3	28.0	15.5		19.5	17.0
4	0	0		24.0	23.0
5	35.0	11.0		10.5	9.0
6	18.0	17.0		23.5	22.5
7	0	0		34.0	37.5
8	3.5	3.0		20.0	21.5
9	23.5	6.5		20.0	21.0
10	18.5	15.5		—	—
11	30.0	11.5			
12	18.5	13.0	S.D.	6.364	7.75
13	15.0	5.0			
14	42.5	26.0	Mean	21.0	21.1
15	15.5	2.0			
16	51.5	20.5	Var.	36.0	53.4
17	21.5	5.0			
	—	—		t = 0.03	
S.D.	14.819	9.767		df = 16	
Mean	22.32	11.559		.975 < p < .980	
Var.	206.67	89.79			
				t = 2.50	
				df = 32	
				p < .02	

⁺ Samples were heat-inactivated at 56 C for 30 min. then divided into two aliquots. One aliquot was immediately tested for cytotoxicity using the microcytotoxicity assays. The second aliquot was stored at -20°C for up to 2 months and tested against fresh target cells obtained from the same individual as the initial serum aliquot was assayed against.

⁺⁺ Samples were divided into aliquots prior to heat-inactivation at 56°C for 30 minutes. One aliquot was heat-inactivated and used immediately in the microcytotoxicity assay. The second aliquot was stored at -20°C for up to two months, thawed, then heat-inactivated and tested for cytotoxic activity using the microcytotoxicity assay. Target cells were obtained from the same individual prior to performing the assay.

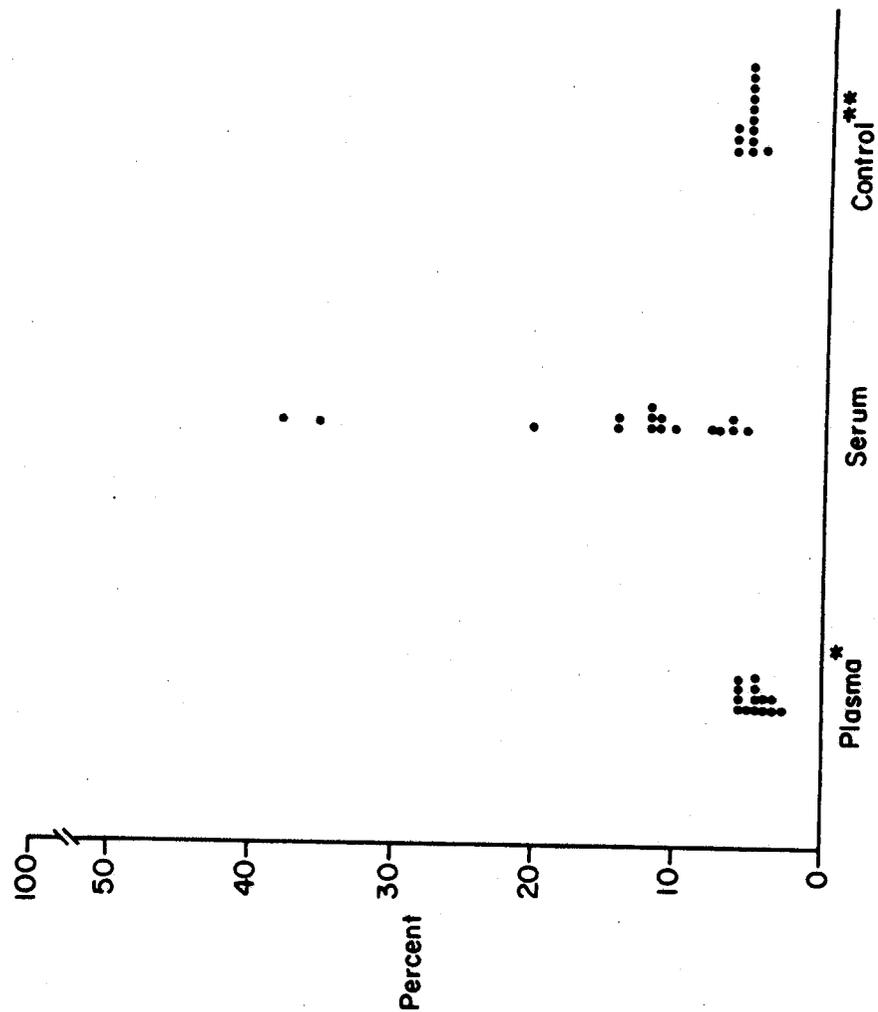
Table 5. Effect of DHF Sera Dilution on Lymphocytotoxicity* Against Unfractionated Lymphocytes from Healthy Thai Adults

Patient	Dilution of sera					
	Neat**	Freeze-thawed	1:2	1:4	1:8	1:16
1	18.5	7.5	8.5	9.0	8.0	6.5
2	24.0	5.5	7.5	10.0	9.0	6.0
3	40.5	15.5	5.5	3.5	0	0
4	21.0	15.5	18.0	17.5	8.0	4.0
5	32.0	16.0	10.0	6.5	6.5	1.0
6	30.5	16.0	6.0	1.5	0	0
7	51.5	20.0	6.0	5.0	4.0	2.5
8	22.0	6.0	4.5	3.5	3.0	1.5

* Average lymphocytotoxicity determined prior to freezing serum at - 20°C

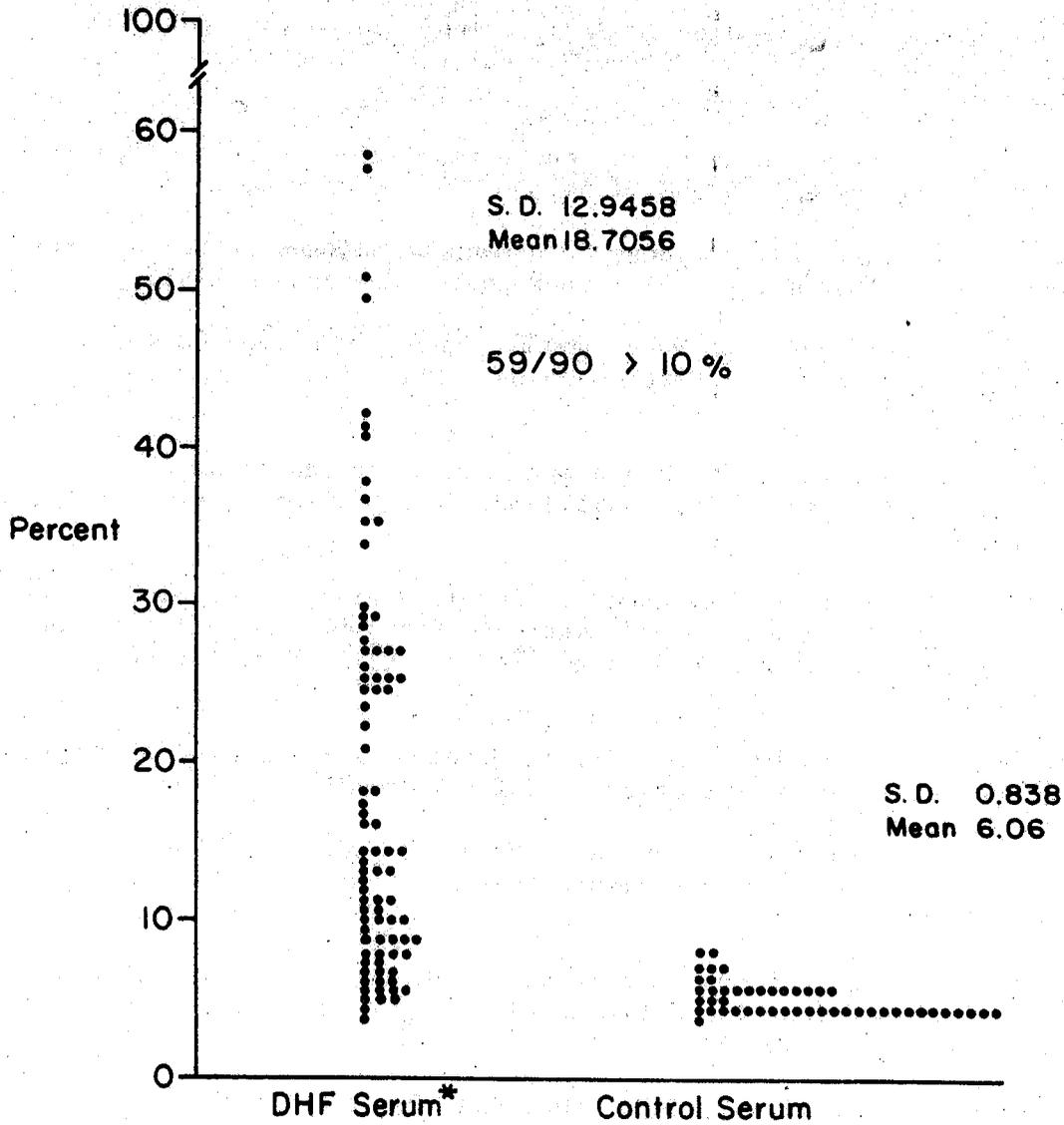
** Test sera diluted with pooled commercial human AB sera (Microbiological Associates, Batch C893018). Assay done at 15°C. No activity detected in assays done at 37°C.

Figure 1. Cytotoxic Activity of DHF Plasma and Serum at 15°C.



* Plasma and Serum obtained from aliquots of the patients blood sample.
 ** Normal control sera from healthy Thai adults.

Figure 2. Death of normal mononuclear cells. (percentage) resulting from incubation of sera from Thai Children Diagnosed as having dengue hemorrhagic fever.



* Obtained on first day of admission, Confirmed DHF Serologically.

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