

COLD-REACTIVE LYMPHOCYTOTOXIC ANTIBODIES IN THAI CHILDREN WITH DENGUE HEMORRHAGIC FEVER

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OBJECTIVE : To determine the target cell population and the chemical nature of lymphocytotoxic antibodies in sera from patients infected with dengue hemorrhagic fever.

INTRODUCTION :

The presence of lymphocytotoxic antibodies in the sera of patients with autoimmune (9), parasitic (10) or viral diseases (4, 7) has been well documented. These lymphocytotoxic antibodies differ from conventional cytotoxins in that they are autocytotoxic, are complement and temperature (15°C) dependent (5, 6) and their presence is independent of allogeneic immunization. The role of lymphocytotoxic antibody in the immuno-pathogenesis or manifestation of the diseases in which they are found is unknown; however, they may interact with cells or other serum factors to modulate the patients' immune response.

Recently, Wells, et al., (11) reported that a major shift occurs within several component cell subpopulations of the immune system in Thai children with DHF. A significant increase in atypical lymphocytes and non-T, non-B, non-Fc receptor bearing Null cells, and a significant decrease in T-cells was present in the peripheral blood obtained from DHF patients on the day of hospitalization.

In addition Boonpucknavig, et al., (1) recently used indirect immunofluorescence to show anti-lymphocyte antibody in the serum of patients with dengue hemorrhagic fever (DHF). However, their study did not determine the incidence of anti-lymphocyte antibodies in the sera of DHF patients and in particular whether or not these antibodies were cytotoxic *in vitro* to autologous and/or allogeneic lymphocytes.

We now report that 49% of sera from Thai children with DHF contain cold-reactive lymphocytes in the presence of complement and which correlate with an increase in the number of polymorphonuclear cells and a decrease in total

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lymphocytes.

MATERIAL AND METHODS..:

Collection and Processing of Specimens : Heparinized and non-heparinized peripheral blood samples were obtained from Thai children examined at the Bangkok Children's Hospital after they were clinically diagnosed as having dengue hemorrhagic fever (DHF) and after informed consent was obtained from the patients' parent or guardian. All patients in this study were classified as having secondary dengue infections according to the serologic criteria of Winter, et al., (12). Heparinized blood obtained from either DHF patients or healthy volunteers was centrifuged over a Ficoll-Hypaque gradient (2) and the resultant mononuclear cells (MNC) were washed three times in Hanks Balanced Salt Solution (HBSS) prior to use as target indicator cells in the lymphocytotoxicity test.

Non-heparinized blood was clotted at room temperature to obtain serum, and serological confirmation of DHF was proven using the hemagglutination inhibition test (HI).

Lymphocytotoxicity Test : The cytotoxicity test was performed, in duplicate, in flat bottom culture trays (Coster Cluster 96, Cambridge, Massachusetts) as previously described (11); 0.1 ml heat-inactivated serum was incubated with 0.1 ml lymphocyte suspension (3×10^6 MNC/ml) for 30 minutes at 4°C, 15°C or 37°C; 0.1 ml fresh rabbit serum as complement source was then added and the mixture incubated for an additional four hours at either 4°C, 15°C or 37°C. The percentage of dead cells was determined by eosin dye inclusion. Healthy Thai adult males served as donors for normal indicator target cells. As controls, MNC were incubated with sera from a panel of healthy donors and rabbit serum, as well as rabbit serum alone. In these experiments, the percentage of dead cells averaged less than 7%. Serum was only classified as positive if cytotoxicity was more than 2 S.D. above the mean control value. Sera from patients with systemic lupus erythematosus or *P. falciparum* malaria, containing high lymphocytotoxic antibody activity, served as positive controls while sera from healthy Thai adults served as negative controls.

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.

Statistics : A two tailed student T test was generally used to determine statistical significance of difference. However, since it was previously hypothesized that patients with lymphocytotoxic antibodies would have lower total lymphocyte counts and be more severely ill than patients lacking detectable lymphocytotoxic antibodies a one tailed student T test was used to determine the statistical significance of difference in these comparisons (Table 3).

RESULTS :

Effect of Temperature on Activity of Serum Lymphocytotoxic Antibodies from Patients with DHF : Sera from patients with DHF were tested against target indicator cells from healthy donors in assays performed at different incubation temperatures (Figure 1). Normal control cells had a killing rate of $6.0\% \pm 0.8\%$ (mean \pm S.D.). Positive lymphocytotoxicity by patient's serum was thus defined as greater than 7.6%. Nine of eleven showed positive cytotoxicity at 4°C , 15 of 19 were positive at 15°C , and 3 of 14 were positive at 37°C . The lymphocytotoxic antibodies were therefore predominately "cold reactive" antibodies.

Percentage of Sera Containing Lymphocytotoxic Antibodies : Forty-one of eighty-three (49.4%) serum specimens from patients with DHF had definite cytotoxic activity (defined as greater than 7.6% killing) against lymphocytes from random donors when the microcytotoxicity assays were done at 15°C using a four hours incubation period (Figure 2). Patients' serum lymphocytotoxic activity ranged from a low level of 4.5% to a high level of 58.5%, while none of the serum specimens from normal donors had higher than 8.5% lymphocytotoxic activity. Furthermore, when 13 individual patient's serum specimens containing high ($> 25\%$) lymphocytotoxic activity were tested against a panel of lymphocytes from 10 normal donors at 4°C and 37°C , seven of the 13 individual serum specimens showed at least 20% cytotoxicity against 50 to 100 percent of the donor target MNC at 4°C while little or no activity was present at 37°C (Table 1).

Autocytotoxicity : In six of 13 cases, the sera of patients with DHF were cytotoxic ($> 7.6\%$) to the patients' own lymphocytes (Table 2). An equivalent level of lymphocytotoxic antibody activity was found when the serum specimens containing antibody were tested against allogeneic lymphocytes from other patients and healthy donors. Thus, sera from patients with DHF which contained antibodies cytotoxic to allogeneic lymphocytes generally contained antibodies against the patients' own lymphocytes as well.

Relationship between Lymphocytotoxic Antibodies and Patients' Clinical Blood Profile : The association of serum lymphocytotoxic antibody with the concentration of the patients' peripheral blood cells, the patients' severity of illness, hematocrit, platelet count, acid phosphatase level or the patients' anti-dengue serum hemagglutination inhibition titer is summarized in Table 3.

The mean concentration of polymorphonuclear cells in patients whose sera contained lymphocytotoxic antibodies was significantly higher ($p < .02$) than the mean concentration in the group of patients whose sera lacked lymphocytotoxic activity. Within the group of patients whose serum contained lymphocytotoxic activity, however, the correlation of percent killing with the total PMN count is not significant ($N = 41$, $r = .122$). As expected the presence of lymphocytotoxic antibody was associated significantly with the severity of illness ($.10 < p < .15$) and with a decrease in lymphocyte counts ($p < .06$). However, the role of the three factors (increased concentration of PMN, decrease lymphocyte counts and the severity of illness) and the appearance

of lymphocytotoxic antibodies in DHF awaits further study. The group of patients whose sera contained lymphocytotoxic antibodies were not statistically different in respect to any other hematological aspect when compared with the groups whose sera lacked lymphocytotoxic activity.

DISCUSSION : In this study, 49% of sera obtained on the first day of hospitalization from Thai children with dengue hemorrhagic fever (DHF) contained lymphocytotoxic antibodies. The antibodies lyse target mononuclear cells in the presence of rabbit complement, are predominately cold reactive, and unlike antibodies appearing after alloimmunization, are cytotoxic against autologous lymphocytes. Similar lymphocytotoxic antibodies have been reported in a variety of diseases (4, 6, 8, 9, 10). Several hypotheses have been suggested to explain why lymphocytotoxic antibodies are formed in viral infections including the proposals that the lymphocytotoxic antibodies may be directed against neoantigens induced by the virus (altered self components), against viral antigens that cross-react with antigens of normal lymphocytes, or against tissue antigens produced by adjuvant or transforming effects of viruses that absorbed to or penetrated into the cells (6).

However, increasing attention is being focused on the concept that lymphocytotoxic antibodies may have an autoregulatory feedback function, reacting with specific lymphocyte subpopulations to modulate the host's immune response. For example lymphocytotoxic antibodies may lyse T-cells or subsets of the T-cells causing the elimination of the component lymphocyte populations. This theory is in accord with the recent observations of decreased T cells (11) in children with DHF. Alternatively, the reduced number of T cells could lead to lymphocytotoxic antibody production through the loss of a suppressor cell regulatory effect. Such a hypothesis would help to explain the high incidence of lymphocytotoxic antibodies in the serum of patients with DHF; if, in fact, as the infection progresses, regulatory cells are decreased and lymphocytotoxic antibodies subsequently appear. Thus, further studies will be required to demonstrate that lymphocytotoxic antibodies present in serum from DHF patients are reactive against T-cells or subsets of T-cells (i.e., suppressor T-cells, helper T cells) or that the presence of lymphocytotoxic antibody in DHF serum is related to an alteration in the proportion of specific lymphocyte subpopulations.

Lymphocytotoxic antibodies may, in fact, not be cytotoxic *in vivo* but may react with cells to regulate antibody production. Cicciarelli, et al., (3) postulated that lymphocytotoxic antibody may react by recognizing immunoglobulins on the surface of B-cells and subsequently modulate antibody production by the B-cells. The anti-T cell antibodies reported by Boonpucknavig, et al., (1) may also function in this matter by reacting with specific T-suppressor or T-helper cell subpopulations at physiological temperatures.

Additionally, antibodies may react with lymphocytes, monocytes, platelets or other body components to induce a variety of toxic effects. Although lymphoid cells may be the best indicator cells for demonstrating serum cytotoxicity *in vitro*, other cells could conceivably be affected both *in vitro* and *in vivo* when exposed to these antibodies.

Therefore, further studies are needed to determine the immunoglobulin nature of the antibody, the target cells which the antibody is directed against, the mechanisms leading to lymphocytotoxic antibody production, the *in vitro* and *in vivo* effects of the antibodies on cell functions, and the association of the antibody with the clinical status of the patients' disease.

Table 1. DHF Serum Lymphocytotoxic Activity Against a Panel of MNC from Health Donors.

		Percent Cytotoxicity													%											
		Mononuclear Cell Population ^a													Cells Killed											
Patient	Temperature	1	2	3	4	5	6	7	8	9	10	11	12	13	4°	37°	4°	37°	4°	37°	4°	37°	4°	37°	4°	37°
1	4°	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	37°	>50	>20	>20	>20	>20	>20	>50	>20	>20	>20	>20	>20	>20	>50	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
3	4°	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	37°	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	4°	>80	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>80	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
6	37°	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	4°	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	37°	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	4°	>20	>20	>20	>20	>10	>20	>50	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
10	37°	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
11	4°	>20	>20	>50	>20	>10	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
12	37°	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	4°	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SLE ^b	37°	>50	>50	>50	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>50	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
Negative Control ^c	4°	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	37°	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

^a Panel of MNC from 10 healthy individual of varying HLA-phenotype. All test sera were negative for cytotoxic antibody when complement was not added. Cytotoxicity determined using eosin dye inclusion on technique.

^b Positive control serum from patient with systemic lupus erythematosus.

^c Serum from healthy individual (negative control).

Table 2. Cytotoxic Activity of DHF Sera Against Different Sources of Target Mononuclear Cells.

Serum**	Mononuclear Target Cells* from Patients with DHF		Allogeneic Healthy Normal Donor MNC
	Autologous	Allogeneic	
1	n.d. ⁺	7.0	6.5
2	28.0	n.d.	35.5
3	18.5	27.0	20.5
4	6.5	10.5	11.5
5	5.5	n.d.	6.5
6	n.d.	19.5	24.0
7	n.d.	11.0	14.0
8	n.d.	14.0	13.5
9	n.d.	6.5	7.0
10	6.5	n.d.	7.5
11	n.d.	6.0	10.0
12	n.d.	23.5	27.0
13	6.5	6.0	5.5
14	9.5	12.0	14.5
15	n.d.	10.0	13.5
16	n.d.	10.5	10.0
17	8.5	n.d.	12.0
18	n.d.	11.0	16.0
19	n.d.	7.5	10.0
20	7.0	n.d.	10.5
21	5.0	n.d.	8.9
22	7.0	9.0	7.5
23	34.0	30.5	38.0
24	n.d.	24.5	21.5
25	8.5	n.d.	14.5

* Assays performed at 15^oC. All values indicate percent cytotoxicity.

** All patient's sera obtained on the first day of hospitalization.

n.d.⁺ Not done. Small amount of serum obtained from the patients prevented testing the serum against all three indicator cell populations.

Table 3. Summary of Lymphocytotoxic Antibody Data.

	Lymphocytotoxin (+) (N = 41) Average/Std. Deviation	Lymphocytotoxin (-) (N = 42) Average/Std. Deviation	Statistical Significance ^a
Age	7.48 ± 2.62	8.49 ± 3.15	N.S.
Grade or severity of illness ^b	2.57 ± 0.73	2.40 ± 0.65	.10 < p < .15 ^c
Total WBC (x 10 ³ /mm ³)	6.92 ± 4.54	5.96 ± 3.12	N.S.
Total PMN (x 10 ³ /mm ³)	4.13 ± 3.62	2.57 ± 1.32	p < .02
Total lymphocytes (x 10 ³ /mm ³)	2.16 ± 1.41	2.82 ± 2.13	p < .06 ^c
Total monocytes (x 10 ³ /mm ³)	0.54 ± 0.37	0.51 ± 0.51	N.S.
Bands	0.36 ± 0.29	0.36 ± 0.40	N.S.
Atypical lymphocytes (x 10 ³ /mm ³)	0.25 ± 0.25	0.22 ± 0.27	N.S.
Platelet count (x 10 ³ /mm ³)	50.73 ± 56.74	37.21 ± 20.70 ^d	N.S.
Hematocrit (x 10 ³ /mm ³)	40.90 ± 5.49	41.14 ± 5.34 ^f	N.S.
HAI titer ^g	10.62 ± 1.39 ^h	10.41 ± 1.15 ^h	N.S.
Acid phosphatase level	1.90 ± 0.59	2.11 ± 0.77	N.S.

^a Determined using two tailed student t-test.

^b Standard grading scheme: I = mild, II = moderate, III = shock, IV = shock, moribund.

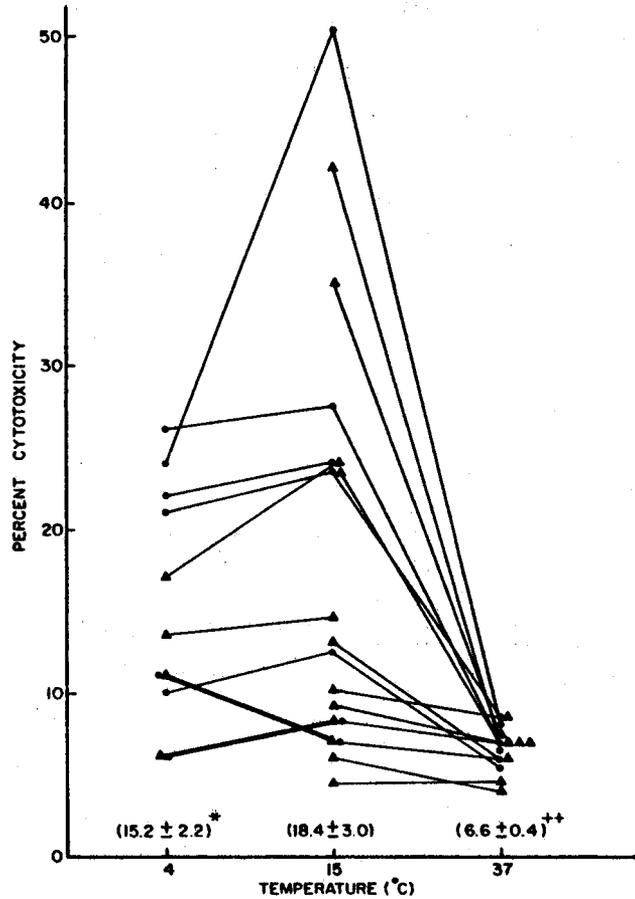
^c Determine using one tailed student t-test; see Material and Methods section.

^d N = 41. ^e N = 35. ^f N = 34.

^g Determined using Log₂ scale (0 = 10, 1 = 1:10, 9 = 1:2560, 10 = 1:5120).

^h N = 40.

Figure 1. Comparison of DHF Lymphocytotoxic Antibody Activity at Different Incubation Temperatures.

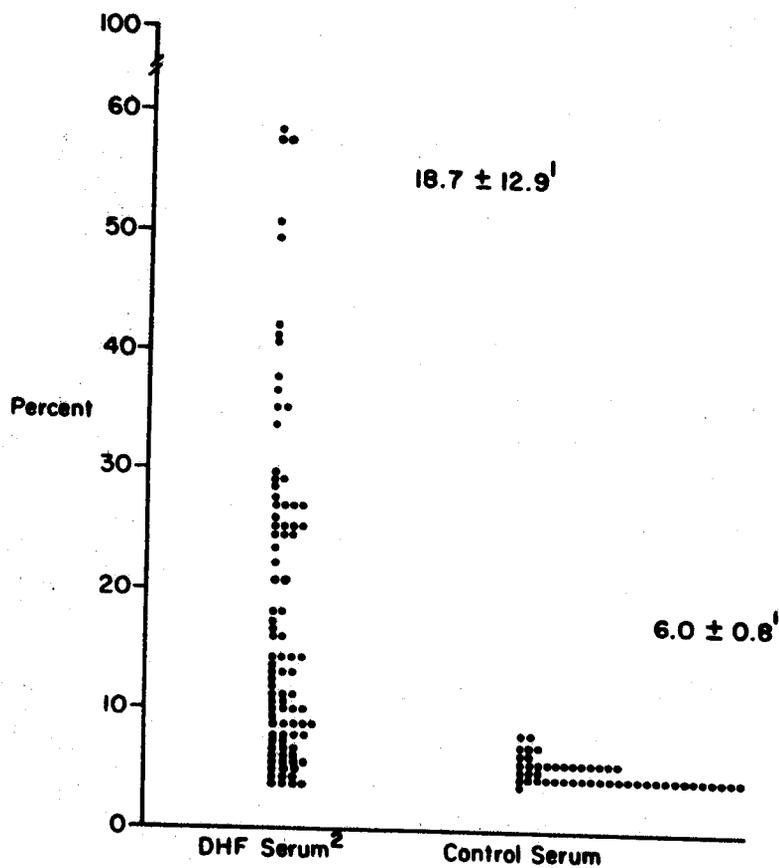


Percent lymphocytotoxicity determined by averaging values from duplicate wells and subtracting control serum cytotoxicity value. (•) Indicates serum samples tested at three temperatures, (▲) indicates serum samples tested only at two temperatures due to the small volume of serum available. Each patient's serum was assayed using allogeneic MNC as target indicator cells.

* Mean lymphocytotoxicity ± standard error of the mean for the sera assayed at the respective temperature.

** Statistically significant difference from cytotoxicity at 15°C (P < .001)

Figure 2. Percentage of normal mononuclear cells lysed after incubation with sera from Thai children with DHF



- 1 :- Mean cytotoxicity \pm standard deviation
 2 :- Serum was obtained on first day of hospitalization. Confirmed DHF Serologically.

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