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Thirteen dengue virus-specific, cytotoxic $CD4^+$ $CD8^-$ T cell clones were established from a donor who was infected with dengue-3 virus. Six patterns of virus specificities were determined. Two serotype-specific clones recognized only dengue-3 virus. Two dengue subcomplex-specific clones recognized dengue-2, -3 and -4 virus, and one subcomplex-specific clone recognized dengue-1, -2, and -3 virus. Four dengue serotype-crossreactive clones recognized dengue-1, -2, -3 and -4 virus. One flavivirus-crossreactive clone recognized dengue-1, -2, -3, and -4 virus and West Nile virus (WNV), but did not recognize yellow fever virus (YFV), while three flavivirus-crossreactive clones recognized dengue-1, -2, -3 and -4 virus, WNV and YFV.

We also examined the recognition of purified NS3 proteins of dengue-3 virus and WNV by these T cell clones. One serotype-specific clone, two dengue subcomplex-specific clones and three dengue serotype-crossreactive clones recognized NS3 of dengue-3 virus. One flavivirus-crossreactive clone recognized NS3 of dengue-3 virus and WNV. These results indicate that

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the NS3 protein contains multiple dominant T cell epitopes.

To determine whether T lymphocytes are activated in vivo during dengue virus infections, we examined the levels of soluble IL-2 receptor (sIL-2R), soluble CD4 (sCD4), soluble CD8 (sCD8) interleukin-2 (IL-2) and interferon gamma (IFNγ), in the sera of patients with DHF/DSS and dengue Fever (DF).

The mean levels of sIL-2R, sCD4, sCD8, IL-2 and IFNγ were significantly higher in the acute sera of patients with DHF/DSS than in the sera of healthy children. The acute sera of patients with DF contained higher levels of sIL-2R, sCD4, IL-2 and IFNγ than the sera of healthy children, but did not have elevated levels of sCD8. The levels of sIL-2R, sCD4 and sCD8 were higher in DHF/DSS than in DF.

We conclude that (i) T lymphocytes are activated and produce IL-2 and IFNγ in vivo during DHF/DSS and DF, (ii) $CD4^+$ T lymphocytes are activated in DHF/DSS and DF, and the level of activation is higher in DHF/DSS than in DF, and (iii) activation of $CD8^+$ T lymphocytes is evident in DHF/DSS, but not in DF.

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HUMAN IMMUNE RESPONSE TO DENGUE INFECTIONS

ANNUAL REPORT

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FOREWORD

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I. INTRODUCTION

Dengue virus infection induces two types of symptoms; dengue fever (DF) and dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) (1,2). DF is a self limited febrile disease, while DHF/DSS is a life threatening disease which is much more commonly observed in secondary infections caused by a serotype of dengue virus that is different from the serotype which caused the primary infection (1,3). The pathogenesis of DHF/DSS is not clearly understood. It has been speculated that augmented dengue virus infection of FcγR-positive monocytes by antibodies to dengue viruses contributes to the pathogenesis (1,2).

We have been studying human T cell responses to dengue viruses, to understand the role of dengue virus-specific human T lymphocytes in the pathogenesis of DHF/DSS and in recovery from dengue virus infections. Dengue virus-specific $CD4^+$ $CD8^-$ T cells and $CD4^-$ $CD8^+$ T cells were detected in subjects after infection with dengue viruses (4,5). $CD8^+$ T cells lyse dengue virus-infected autologous cells in an HLA class I-restricted fashion and recognize E and non-structural proteins (2). $CD4^+$ T cells proliferate and produce IFNγ, which upregulates expression of FcγRI and augments dengue virus infection in the presence of antibody to dengue viruses (6). Based on these results we hypothesized that during secondary infections the number of dengue virus-infected monocytes is increased by infection with dengue virus antibody complexes and by IFNγ which are produced by dengue virus-specific $CD4^+$ T cells, and that lysis of these dengue virus-infected monocytes by $CD4^+$ cytotoxic T lymphocytes (CTL) and $CD8^+$ CTL may lead to DHF/DSS (7,8). To further characterize dengue virus-specific $CD4^+$ T cells, we have established $CD4^+$ $CD8^-$ clones from a donor who had been immunized earlier with YF vaccine and was infected with dengue-3 virus (5). These T cell clones produced IFNγ and lysed dengue Ag-cultured autologous cells. In this report we describe the virus- and dengue serotype-specificity and HLA restriction of these clones.

II. RESULTS

A. Dengue virus-specific, HLA class II-restricted cytotoxic T lymphocytes (CTL)

A-1. Virus- and dengue serotype-specificity of $CD4^+$ T cell clones

Dengue virus-specific T cell clones were established from lymphocytes of donor A using limiting dilution methods as described previously. Seven clones were established using dengue-3 Ag (5), and six clones were established using dengue-2 Ag. Phenotypic analyses using monoclonal antibodies showed that all the clones have $CD3^+$, $CD4^+$ and $CD8^-$ phenotypes.

These clones were examined for their virus- and dengue serotype-specificities in cytotoxic activities (Table 1). JK21 and JK37 lysed target cells cultured with dengue-3 Ag, but did not lyse target cells cultured with dengue-1, -2, -4, YF or WNV Ag. Therefore, they are dengue serotype-specific. JK36 and JK46 lysed target cells cultured with dengue-2, -3 and -4, but did not lyse target cells cultured with dengue-1, YFV or WNV Ag. JK44 lysed target cells cultured with dengue-1, -2, and -3, but did not lyse target cells cultured with dengue-4, YFV or WNV Ag. Therefore, these three clones are dengue subcomplex-specific. Four clones, JK32, JK34, JK39 and JK41, lysed target cells cultured with dengue Ag of four serotypes, but did not lyse target cells cultured with YFV or WNV Ag. Therefore, they are dengue serotype-crossreactive.

JK28 lysed target cells cultured with dengue Ag of four serotypes and YFV Ag, but did not lyse target cells cultured with WNV Ag. JK26, JK43 and JK49 lysed target cells cultured with dengue Ag of four serotypes, YFV and WNV Ag. Therefore, these clones are flavivirus-crossreactive. To confirm the flavivirus-crossreactivity of JK43 at a clonal level, we established subclones from JK43 using a limiting dilution of 0.3 cells per well. Subclones JK43c and JK43d, lysed target cells cultured with dengue Ag of four serotypes, YFV and WNV Ag (Table 2), confirming that JK43 is flavivirus-crossreactive at a clonal level.

The results shown in Tables 1 and 2 indicate that dengue virus-specific $CD4^+$ T cells are heterogeneous in virus- and dengue serotype-specificity, and that there are at least six patterns of specificities.

Table 1. Virus- and dengue serotype-specificity of $CD4^+$ $CD8^-$ T cell clones established from donor A infected with dengue-3 virus[a]

	% specific ^{51}Cr release							
Clones	D-1	D-2	D-3	D-4	YF	WN	Control Ag	No Ag
Dengue serotype-specific								
JK21	1	0	20	0	0	0	0	0
JK37	1	0	57	0	0	0	0	0
Dengue subcomplex-specific								
JK36	0	55	50	15	0	0	0	0
JK46	1	68	67	27	0	0	0	0
JK44	24	65	34	0	0	0	0	0
Dengue serotype-crossreactive								
JK32	52	65	66	55	7	8	10	10
JK34	28	54	52	23	0	0	0	0
JK39	39	62	67	31	0	0	0	0
JK41	12	43	39	12	0	0	0	0

Flavivirus-crossreactive								
JK28	24	46	41	25	0	23	0	0
JK26	23	40	41	19	19	55	0	0
JK43	22	31	34	27	37	43	0	0
JK49	34	60	57	26	29	50	0	0

a. 2.5×10^3 target cells were incubated with effector cells for 6 hours. Percent specific ^{51}Cr release was calculated by the formula described in Materials and Methods. Effector/target ratio was 3:1 for JK21 and JK43, 4:1 for JK41, 6:1 for JK37, JK36, JK46, JK44, JK32, JK34, JK39 and JK49, 7:1 for JK26, and 12:1 for JK28.

Table 2. Flavivirus-crossreactive cytotoxic activity of subclones of JK43[a]

	% specific ^{51}Cr release							
Clones	D-1	D-2	D-3	D-4	YF	WN	Control Ab	No Ab
JK43c	60	59	65	51	76	75	0	0
JK43d	36	54	54	49	71	70	0	0

a. 2.5×10^3 target cells were incubated with effector cells for 6 hours. Percent specific ^{51}Cr release was calculated by the formula described in Materials and Methods. Effector/target ratio was 1:1 for JK43c and 3:1 for JK43d.

A-2. HLA-restriction in the lysis of target cells by $CD4^+$ T cell clones

HLA-restriction of the lysis of target cells by dengue virus-specific $CD4^+$ T cell clones were examined using monoclonal antibodies to HLA molecules (Table 3). Monoclonal Ab to HLA DP inhibited the lysis of target cells by a dengue-3-specific clone JK37, dengue serotype-crossreactive clones JK32, JK34, JK39 and JK41, and flavivirus-crossreactive clones JK26, JK28 and JK49. Monoclonal Ab to HLA DQ inhibited the lysis of target cells by dengue subcomplex-specific clones JK36 and JK46. Monoclonal Ab to HLA DR inhibited the lysis of target cells by a dengue-3-specific clone JK21 and a subcomplex-specific clone JK44. Interestingly, the lysis of target cells by a flavivirus-crossreactive clone JK43 was not inhibited by any of the 3 monoclonal Abs to HLA class II and an antibody to HLA class I. However, the lysis by JK43 was inhibited by a mixture of anti-HLA DP, HLA DQ and HLA DR Abs, and by an antibody to CD3 (data not presented).

These results indicated that dengue virus-specific $CD4^+$ T cell clones are HLA class II-restricted and that HLA DP, DQ and DR are used as restriction elements by the various clones.

Table 3. HLA restriction of lysis of dengue-3 Ag-cultured target cells by $CD4^+$ T cell clones[a]

	% specific ^{51}Cr release			
	None	Anti-HLA DP	Anti-HLA DQ	Anti-HLA DR
Dengue serotype-specific				
JK21	22	21	26	6
JK37	55	5	48	26
Dengue subcomplex-specific				
JK36	25	26	4	20
JK46	45	47	8	41
JK44	51	46	54	14
Dengue serotype-crossreactive				
JK32	83	7	80	71
JK34	87	1	83	77
JK39	46	1	48	45
JK41	56	7	47	40
Flavivirus-crossreactive				
JK28	91	11	78	77
JK26	64	1	64	69
JK43	56	49	55	59
JK49	53	1	41	57

a. 2.5×10^3 target cells were incubated with effector cells for 6 hours in the presence of monoclonal antibodies at final dilution of 1:80. B7/21.7, S3/4 and OKIa1 were used as anti-HLA DP, anti-HLA DQ and anti-HLA DR, respectively. Effector/target ratio was 4:1 for JK43, 5:1 for JK21, JK46 and JK39, 6:1 for JK37, JK44, JK32, JK26 and JK49, 7:1 for JK36, 11:1 for JK28, 14:1 for JK34, and 15:1 for JK41.

A-3. Recognition of NS3 by dengue virus-specific T cell clones

We have reported that NS3 induces high levels of proliferation responses of donor A PBMC in bulk cultures (9). We tried to determine whether $CD4^+$ T cell clones recognize NS3 protein (Table 4). Dengue-3-specific clone JK37, subcomplex-specific clones JK36 and JK46, and serotype-crossreactive clones JK32, JK34 and JK39, lysed target cells cultured with NS3 obtained from dengue-3 infected cells, but did not lyse target cells cultured with NS1 obtained from dengue-3 virus-infected cells or NS3 from WNV-infected cells. A flavivirus-crossreactive clone JK43 lysed target cells cultured with purified NS3 obtained from dengue-3 infected cells or WNV-infected cells, but did not lyse target cells cultured with NS1 obtained from dengue-3 virus-infected cells.

Table 4. Recognition of NS3 protein by dengue virus-specific $CD4^+$ T cell clones[a]

	% specific ^{51}Cr release						
	D-3[b] NS1	D-3 NS3	WN NS3	Control cell protein	D-3 Ag	WN Ag	No Ag
Dengue serotype-specific							
JK37	2	13	2	ND	56	0	1
Dengue subcomplex-specific							
JK36	10	40	ND	15	87	ND	19
JK46	7	20	0	ND	75	ND	7
JK44	0	1	0	ND	47	0	0
Dengue serotype-crossreactive							
JK32	11	27	10	14	77	10	6
JK34	2	14	0	0	79	0	0
JK39	7	19	0	ND	42	ND	6
JK41	0	3	ND	4	66	ND	0
Flavivirus-crossreactive							
JK28	2	7	0	1	70	30	0
JK26	0	5	0	0	60	49	0
JK43	7	25	84	8	78	93	0
JK49	7	5	0	2	77	72	0

a. 2.5×10^3 target cells were incubated with effector cells for 6 hours. Effector/target ratio was 4:1 for JK32, 6:1 for JK37, JK44, JK39 and JK26, 7:1 for JK46, 8:1 for JK41 and JK43, 4:1 for JK49, 10:1 for JK34 and JK28, and 11:1 for JK36.

b. Autologous EBV-transformed cells were cultured with dengue-3 NS1, dengue-3 NS3, West Nile NS3 and control cell protein at final concentration of 20 ug/ml, and dengue-3 Ag and West Nile virus Ag at 1:80 for 24 hours.

Lysis of target cells cultured with NS3 of dengue-3 by JK34 and JK39 was inhibited by antibody to HLA DP (Table 5). JK44, JK41, JK26, JK28 and JK49 did not lyse target cells cultured with NS1 or NS3 proteins (Table 4). These results suggest that the NS3 protein contains multiple epitopes recognized by dengue virus-specific $CD4^+$ T cells of various serotype-specificities.

Table 5. HLA DP-restricted lysis of NS3-cultured target cells by JK34 and JK39[a]

	% specific ^{51}Cr release			
	None	Anti-HLA DP	Anti-HLA DQ	Anti-HLA DR
JK34	11	0	15	10
JK39	31	10	32	27

a. 2.5×10^3 target cells were incubated with effector cells for 6 hours in the presence of monoclonal antibodies at final dilution of 1:80. Effector/target ratio was 10:1 for JK34 and JK39.

B. Activation of T lymphocytes in vivo in dengue virus infections

Activated T lymphocytes secrete interleukin 2 (IL-2) (10,11), IFNγ(11), and granulocyte-macrophage colony stimulating factor (GM-CSF) (12,13), and release soluble interleukin 2 receptor (sIL-2R) (14), soluble CD8 (sCD8) (15,16) and soluble CD4 (Susan Kline, personal communication). Monocytes/macrophages produce interleukin 1 (IL-1) (17) and tumor necrosis factorα (TNFα) (18,19). In collaboration with Dr. Bruce L. Innis, Dr. Ananda Nisalak (Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand) and Dr. Suchitra Nimmannitya (Children's Hospital, Bangkok, Thailand), we have begun to analyze the activation of T lymphocytes and monocytes in vivo by measurements of cytokines and soluble cell surface proteins in the sera of Thai children with DHF/DSS and DF. We also analyzed sera of Thai children with febrile illnesses other than DHF/DSS, DF or Japanese encephalitis and sera of healthy Thai children.

B-1. Levels of sIL-2R in the sera of dengue patients

The sera from 37 patients with DHF/DSS (3 primary and 34 secondary infections) were examined for sIL-2R levels (Table 6 and Figure 1). The levels of sIL-2R were significantly higher in the sera of patients with DHF/DSS on days 1-3 after admission than in the sera of healthy Thai children ($p<0.001$). The sIL-2R levels on days 7-10 were reduced from days 1-3, but were still significantly higher than levels in healthy children ($p<0.001$).

The sera from 34 patients with DF (3 primary and 31 secondary infections) were examined. SIL-2R levels in the sera obtained from patients with DF in the acute and in the convalescent stages were significantly higher than levels in healthy children ($p<0.001$) (Figure 1 and Table 1). The acute sera of patients with DHF/DSS contain significantly higher levels of sIL-2R than the acute sera of patients with DF ($p<0.05$) (Table 6).

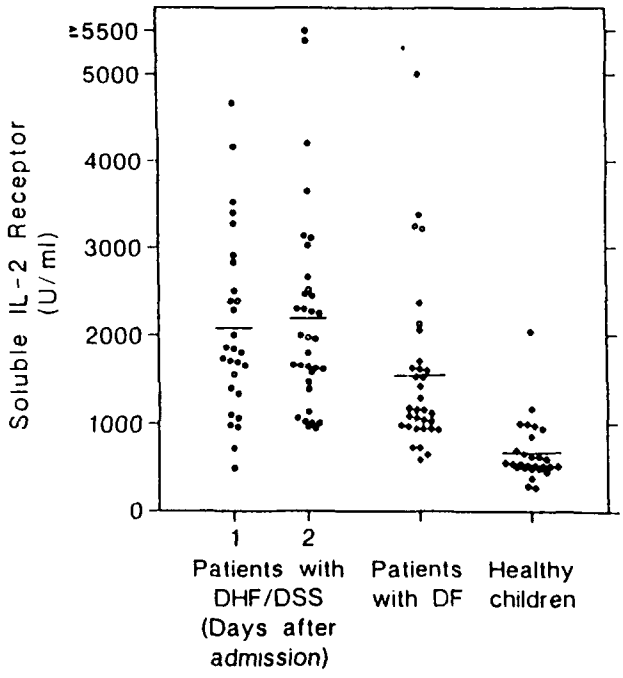
Table 6. Levels of sIL-2R in the sera from patients with DHF/DSS and DF.

Sera	Days	Average Titers ± S.D. (U/ml)	(Number of Donors)	p-values	
DHF/DSS	day 1	2079±1036	(28)	<0.001[a]	<0.05[b]
	day 2	2187±1221	(34)	<0.001	<0.02
	day 3	2126±1325	(23)	<0.001	NS
	days 7-10	1191±574	(32)	<0.001	
Dengue fever	Acute	1536±957	(33)	<0.001	--
	Convalescent	1052±405	(34)	<0.001	
Uncharacterized febrile diseases	Acute	1079±383	(25)	<0.001	
	Convalescent	904±202	(26)	<0.005	
Healthy children		665±347	(28)	--	

[a]Titers of sIL-2R were compared with the titers in the sera of healthy Thai children.
[b]Titers of sIL-2R were compared with the titers in acute sera of patients with DF. NS denotes not significant (p>0.05).

Figure 1

Levels of soluble IL-2 receptor (sIL-2R) in the acute sera of patients with dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) and with dengue fever (DF). The arithmetic mean titers of sIL-2R were 665 U/ml (n=28) in the sera of healthy Thai children, 2079 U/ml (n=28), p<0.001) on day 1 and 2187 U/ml (n=35, p<0.001) on day 2 in the sera of patients with DHF/DSS, and 1536 U/ml (n=33), p<0.001) in the acute sera of patients with DF.



B-2. Levels of sCD4 in the sera of dengue patients

The sera from 30 patients with DHF/DSS (2 primary and 28 secondary infections) were examined for sCD4 (Table 7 and Figure 2). The levels of sCD4 on days 1-3 were significantly higher than the levels in the sera of healthy children ($p<0.001$). The levels decreased by days 7-10 to those seen in healthy children. SCD4 levels in 21 patients with DF (5 primary and 16 secondary infections) in the acute stage were higher than those in healthy children ($p<0.01$) (Table 7 and Figure 2). The sCD4 levels on days 1-3 in patients with DHF/DSS were significantly higher than the sCD4 levels in the acute sera of patients with DF ($p<0.005$ on day 1, $p<0.001$ on day 2, and $p<0.01$ on day 3) (Table 7).

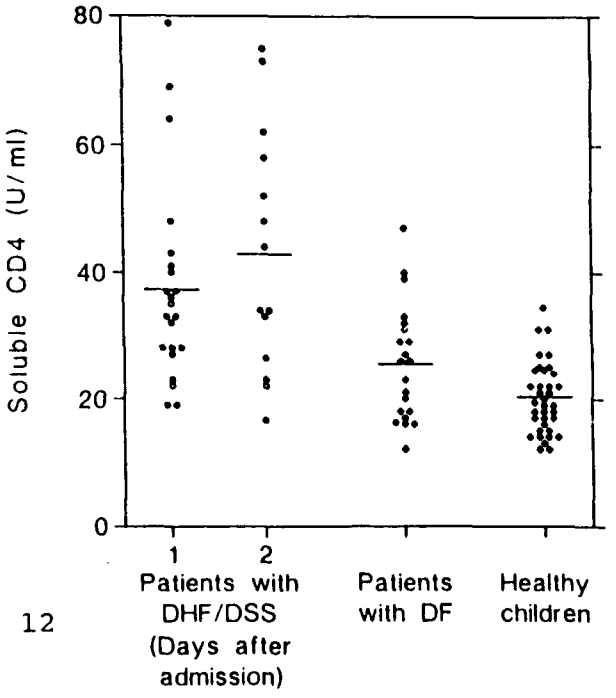
Table 7. Levels of sCD4 in the sera from patients with DHF/DSS and DF.

Sera	Days	Average Titers ± S.D. (U/ml)	(Number of Donors)	p-values	
DHF/DSS	day 1	37.3±15.7	(22)	<0.001[a]	<0.005[b]
	day 2	42.9±19.0	(14)	<0.001	<0.001
	day 3	39.0±16.9	(11)	<0.001	<0.01
	days 7-10	19.0±11.2	(6)	NS	
Dengue fever	Acute	25.5±9.2	(21)	<0.01	--
	Convalescent	22.0±8.6	(11)	NS	
Healthy children		20.4±5.4	(40)	--	

[a]Titers of sCD4 were compared with the titers in the sera of healthy Thai children. NS denotes not significant ($p>0.05$).
[b]Titers of sCD4 were compared with the titers in acute sera of patients with DF.

Figure 2

Levels of sCD4 in the acute sera of patients with DHF/DSS and DF. The arithmetic mean titers of sCD4 were 20.4 U/ml (n=40) in the sera of healthy Thai children, 37.3 (n=22, $p<0.001$) on day 1, 42.9 U/ml (n=14, $p<0.001$) on day 2 in the sera of patients with DHF/DSS, and 25.5 U/ml (n=21, $p<0.01$) in the acute sera of patients with DF.



B-3. Levels of sCD8 in the sera of dengue patients

SCD8 levels were significantly higher in the sera of 42 patients with DHF/DSS (3 primary and 39 secondary infections) on days 1-3 than in the control sera (Table 8) ($p<0.001$). The levels of sCD8 decreased by days 7-10, but were still higher than levels in healthy children ($p<0.001$). The average sCD8 levels in 37 patients with DF (7 primary and 30 secondary infections) in acute and convalescent stages were similar to those in healthy children (Table 8 and Figure 3). However, high levels of sCD8 were detected in the acute sera of 4 hospitalized patients with DF (average: 1516 $\pm$ 743 U/ml). SCD8 levels on days 1-3 in patients with DHF/DSS were significantly higher than the sCD8 levels in the acute sera of patients with DF ($p<0.001$) (Table 8).

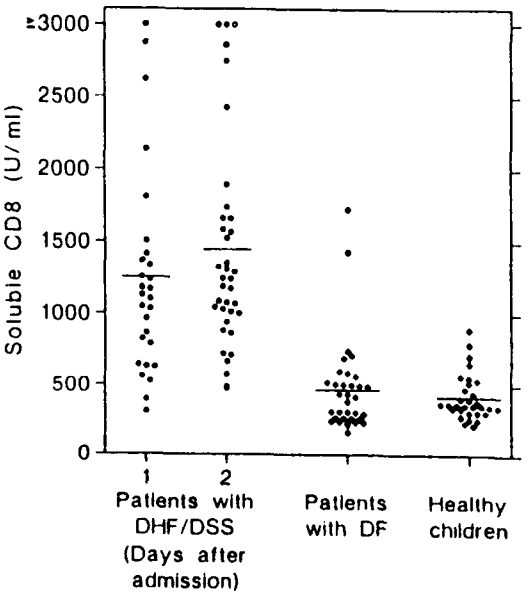
Table 8. Levels of sCD8 in the sera from patients with DHF/DSS and DF.

Sera	Days	Average Titers + S.D. (U/ml)	(Number of Donors)	p-values	
DHF/DSS	day 1	1252±780	(28)	<0.001[a]	<0.001[b]
	day 2	1447±862	(37)	<0.001	<0.001
	day 3	1555±877	(26)	<0.001	<0.001
	days 7-10	928±375	(10)	<0.001	
Dengue fever	Acute	519±445	(36)	NS	--
	Convalescent	389±300	(32)	NS	
Uncharacterized febrile diseases	Acute	372±125	(26)	NS	
	Convalescent	433±158	(26)	NS	
Healthy children		416±164	(31)	--	

[a]Titers of sCD8 were compared with the titers in the sera of healthy Thai children. NS denotes not significant ($p>0.05$).
[b]Titers of sCD8 were compared with the titers in the acute sera of patients with DF.

Figure 3

Levels of sCD8 in the acute sera of patients with DHF/DSS and DF. The arithmetic mean titers of sCD8 were 416 U/ml (n=31) in the sera of healthy Thai children, 1252 U/ml (n=28, $p<0.001$) on day 1, 1447 U/ml (n=37, $p<0.001$) on day 2 in the sera of patients with DHF/DSS, and 519 U/ml (n=36, not significant) in the acute sera of patients with DF.



B-4. Levels of IL-2 in the sera of dengue patients

The titers of IL-2 in the sera of 41 patients with DHF/DSS (3 primary and 38 secondary infections) on days 1, 2 and 3 were significantly higher (Table 9 and Figure 4) than those in control sera ($p<0.001$). IL-2 levels greater than 10 U/ml were detected in 58% (22/38), 57% (21/37) and 50% (13/26) of the patients' sera on days 1, 2 and 3, respectively, while only 2 of 28 sera of healthy children contained greater than 10 U/ml of IL-2 ($p<0.001$ on days 1-3 by Chi-square test). 50% (18/36) of the sera of patients with DHF/DSS still contained IL-2 levels above 10 U/ml on days 7-10 ($p<0.001$ by Chi-square test). The average titer on days 7-10 was significantly higher than that in the control sera ($p<0.001$).

Sera of the patients with DF also contained significantly higher levels of IL-2 in the acute and convalescent sera than the sera of normal Thai children ($p<0.001$) (Table 9 and Figure 4). IL-2 levels greater than 10 U/ml were detected in 67% (24/36) and 72% (26/36) of the sera in acute and convalescent stages, respectively ($p<0.001$ in both stages by Chi-square test). The IL-2 levels were similar in the acute sera of patients with DF and in the sera of patients with DHF/DSS on day 1-3 (Table 9).

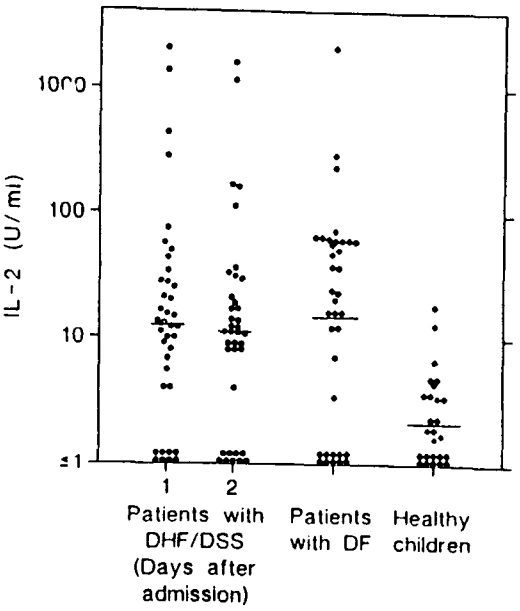
Table 9. Levels of IL-2 in the sera from patients with DHF/DSS and DF.

Sera	Days	Average Titers ±S.D. (Log_{10} U/ml)	(Number of Donors)	p-values	
DHF/DSS	day 1	1.095±0.781	(38)	<0.001[a]	NS[b]
	day 2	1.044±0.811	(37)	<0.001	NS
	day 3	1.017±0.891	(26)	<0.001	NS
	days 7-10	1.149±1.153	(36)	<0.001	
Dengue fever	Acute	1.167±0.869	(36)	<0.001	--
	Convalescent	1.686±1.982	(36)	<0.001	
Uncharacterized febrile diseases	Acute	1.081±1.008	(26)	<0.001	
	Convalescent	2.194±1.215	(26)	<0.001	
Healthy children		0.328±0.360	(28)	--	

*Titers of IL-2 (Log_{10}) were compared with the titers in the sera of healthy Thai children.
**Titers of IL-2 (Log_{10}) were compared with the titers in the acute sera of patients with DF. NS denotes not significant ($p>0.05$)

Figure 4

Levels of IL-2 in the acute sera of patients with DHF/DSS and DF. The geometric mean titers of IL-2 were 2.1 U/ml (n=28) in the sera of healthy Thai children, 12.5 U/ml (n=38, $p<0.001$) on day 1 and 11.1 U/ml (n=37, $p<0.001$) on day 2 in the sera of patients with DHF/DSS, and 14.7 U/ml (n=36, $p<0.001$) in the acute sera of patients with DF.



B-5. Levels of IFNγ in the sera of dengue patients

The sera of 20 patients with DHF/DSS (1 primary and 19 secondary cases) were examined for IFNγ (Table 10 and Figure 5). IFNγ was detected in all the tested sera (20/20) on day 1, in 95% (19/20) on day 2 and in 85% (11/13) on day 3, while it was detected in 13% (4/30) of control sera ($p<0.001$ on days 1-3 by Chi-square test). Titers of IFNγ in the sera of patients with DHF/DSS on days 1-3 were higher than those in the control sera ($p<0.001$). The titers decreased gradually, but were still higher than those in control sera on days 7-10 ($p<0.001$) when 65% (13/20) of sera still contained detectable levels of IFN ($p<0.001$ by Chi-square test) (Table 10).

IFNγ levels in acute ($p<0.001$) and convalescent ($p<0.002$) sera of patients with DF were significantly higher than the levels in the sera of healthy children (Table 10). IFNγ was detected in 91% (30/33) and 55% (18/33) of the sera in acute and convalescent stages, respectively ($p<0.001$ in acute stage and $p<0.01$ in convalescent stage). IFNγ levels in the acute sera of patients with DF was similar to the IFNγ levels in patients with DHF/DSS on day 1 (Table 10).

Table 10. Levels of IFNγ in the sera from patients with DHF/DSS and DF.

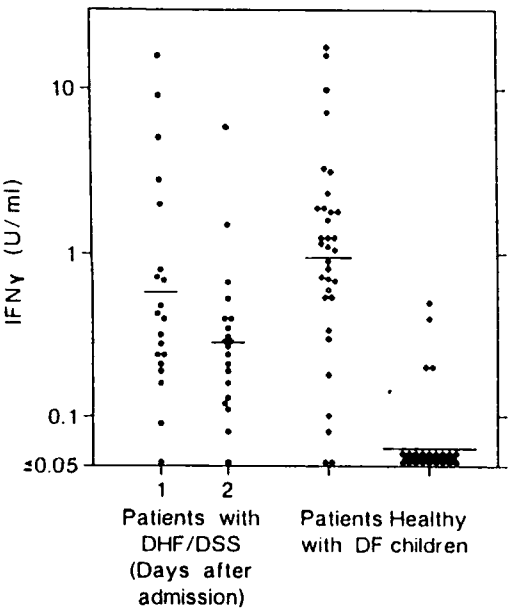
Donors with	Days	Average Titers ± S.D. (Log_{10} U/ml)	(Number of Donors)	p-values
DHF/DSS	day 1	-0.235±0.658	(20)	<0.001[a] NS[b]
	day 2	-0.544±0.455	(20)	<0.001 <0.005
	day 3	-0.700±0.353	(13)	<0.001 <0.001
	days 7-10	-0.910±0.345	(20)	<0.005

Dengue fever	Acute	-0.022±0.646	(33)	<0.001	--
	Convales.	-0.869±0.433	(33)	<0.002	
Uncharacterized febrile diseases	Acute	-0.173±0.536	(26)	<0.001	
	Convales.	-0.760±0.506	(26)	<0.001	
Healthy children		-1.197±0.277	(30)	--	

[a]Titers of IFN (Log_{10}) were compared with the titers in the sera of healthy Thai children.
[b]Titers of IFN (Log_{10}) were compared with the titers in the acute sera of patients with DF. NS denotes not significant ($p<0.05$).

Figure 5

Levels of IFNγ in the acute sera of patients with DHF/DSS and DF. The geometric mean titers of IFN were 0.064 U/ml (n=30) in the sera of healthy Thai children, 0.58 U/ml (n=20, p<0.001) on day 1 and 0.29 U/ml (n=20, p<0.001) on day 2 in the sera of patients with DHF/DSS, and 0.95 U/ml (n=20, p<0.001) in the acute sera of patients with DF.



B-6. GM-CSF, IL-1α and TNFα in sera of patients with DHF/DSS

We then examined the titers of GM-CSF, IL-1α, and TNFα in the sera from patients with DHF/DSS. GM-CSF is known to be secreted by T cells (12,13). IL-1α and TNFα are known to be secreted by monocytes/macrophages (16-18). The levels of GM-CSF in the sera of patients with DHF/DSS on days 1, 2, 3 and 7-10 were similar to the levels in the sera of healthy children (Figure 6). The titers of IL-1α were slightly higher in the sera of patients with DHF/DSS than in the sera of healthy children, but the difference was not statistically significant on days 1-3 (Figure 7). Interestingly, the titers of IL-1α in the sera of DHF/DSS patients were higher on days 7-10 than those in the control sera ($p<0.02$). TNFα was not detected in sera available from 18 DHF/DSS patients between days 1-11. These results indicate that the serum levels of monokines were not elevated during acute stage of DHF/DSS.

Figure 6

Levels of GM-CSF in the sera of patients with DHF/DSS. The arithmetic mean titers of GM-CSF were 50.7 pg/ml (n=28) in the sera of healthy Thai children, 54.5 pg/ml (n=14, not significant (NS)) on day 1, 38.6 pg/ml (n=16, NS) on day 2, 56.9 pg/ml (n=10, NS) on day 3, and 54.4 pg/ml (n=15, NS) on days 7-10 in the sera of patients with DHF/DSS.

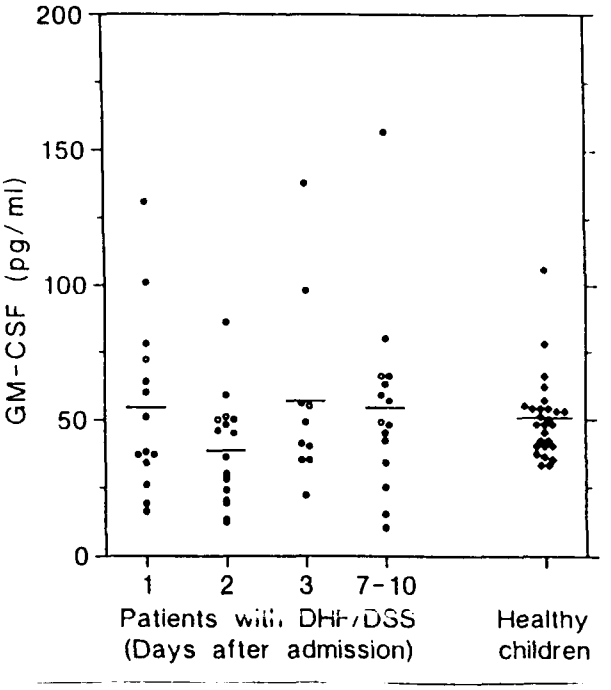
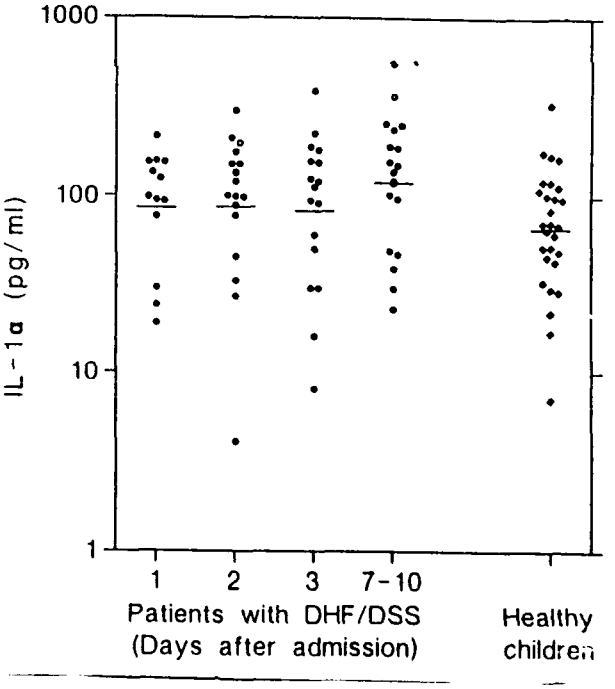


Figure 7

Levels of IL-1α in the sera of patients with DHF/DSS. The geometric mean titers of IL-1 were 66.1 pg/ml (n=28) in the sera of healthy Thai children, 84.9 pg/ml (n=13, not significant (NS)) on day 1, 86.5 pg/ml (n=17, NS) on day 2, 82.2 pg/ml (n=17, NS) on day 3 and 119.9 pg/ml (n=19, $p<0.02$) on days 7-10 in the sera of patients with DHF/DSS.



C. Dengue-2 virus infection of human mononuclear cell lines and establishment of persistent infections

Study of dengue virus-PBMC interaction is important to understand the pathogenesis of dengue virus infections. Many human mononuclear cell lines are available; therefore, we decided to examine a variety of human mononuclear cell lines to determine whether they could be infected with dengue virus and be useful for future studies. In this paper we attempt to infect 23 human mononuclear cell lines, including ten myelomonocytic cell lines, eight B cell lines and five T cell lines with dengue-2 virus. All the cell lines can be infected with dengue-2 virus. Persistent dengue-2 virus infection is established using K562 (myelomonocytic), Raji (B) and HSB-2 (T) cell lines.

C-1. Acute infection of human mononuclear cell lines with dengue-2 virus

Ten myelomonocytic cell lines, eight B cell lines and five T cell lines were used in the experiments. All the cell lines could be infected with dengue-2 virus in the absence of antibody (Table 11). Antibody to dengue-2 virus augmented dengue-2 virus infection of myelomonocytic cell lines determined by IF and virus assays. However, antibody did not augment infection of B or T cell lines. In myelomonocytic cell lines K562, HEL92-1-7, JOSK-I and JOSK-M cells contained a high percentage of antigen-positive cells, while HL-60, KG-1 and THP-1 contained fewer antigen-positive cells. In B cell lines Jiyoye, ARH-77 and IM-9 contained a high percentage of antigen-positive cells, while Ramos, Daudi and CA46 contained fewer antigen-positive cells. In T cell lines Jurkat and CEM contained a high percentage of antigen-positive cells.

Table 11: Dengue virus infection of human mononuclear cell lines[a]

		% dengue-2 antigen-positive cells			Virus titer (p.f.u./ml)
Cell line	Antibody[b]	24 hrs	48 hrs	72 hrs	at 48 hrs
Myelomonocytic cell lines					
K562	+	90.8	95.9	99.0	5.0×10^6
	-	57.8	76.5	99.0	4.5×10^6
HEL92-1-7	+	35.8	35.4	41.8	2.3×10^5
	-	22.8	26.5	37.2	1.5×10^5
JOSK-I	+	37.5	34.3	18.5	5.0×10^5
	-	7.7	4.1	1.7	5.7×10^4
JOSK-M	+	28.6	23.0	8.4	2.3×10^5
	-	16.0	11.0	3.5	2.3×10^5

JOSK-S	+	16.1	9.5	6.4	1.6×10^5
	-	6.3	3.8	3.4	1.5×10^5
JOSK-K	+	15.1	14.1	3.9	4.0×10^5
	-	3.6	3.7	3.3	1.6×10^5
U937	+	15.7	17.4	4.1	3.8×10^4
	-	0.6	1.3	0.9	4.2×10^3
THP-1	+	7.5	6.8	9.8	1.1×10^5
	-	0.4	0.5	0.5	2.0×10^4
KG-1	+	2.4	1.7	0.9	1.3×10^4
	-	1.4	1.3	0.5	3.0×10^3
HL-60	+	1.7	2.9	1.6	1.4×10^3
	-	0.2	0.4	0.3	3.5×10^2
B cell lines					
Jiyoye	+	82.6	81.4	81.7	4.0×10^4
	-	82.4	86.4	85.0	5.0×10^4
ARH-77	+	28.4	45.7	30.8	5.4×10^5
	-	25.0	30.9	31.4	3.9×10^5
IM-9	+	22.6	22.7	17.2	3.5×10^5
	-	30.3	25.3	13.1	3.1×10^5
Raji	+	10.2	6.6	11.7	8.5×10^3
	-	7.8	9.6	13.7	9.5×10^3
HS-Sultan	+	8.8	6.9	8.3	4.0×10^3
	-	4.8	5.6	7.5	3.0×10^3
CA46	+	1.5	1.5	1.3	4.0×10^3
	-	2.5	2.2	1.0	2.0×10^3
Daudi	+	1.1	1.1	0.8	1.0×10^2
	-	0.5	1.8	0.8	1.0×10^2
Ramos	+	0.8	0.5	0.4	8.0×10^0
	-	0.5	0.5	0.3	5.0×10^0
T cell lines					
Jurkat	+	50.0	39.2	39.7	6.6×10^4
	-	48.7	44.5	34.5	6.1×10^4
CEM	+	36.3	27.0	21.3	1.3×10^5
	-	34.0	23.4	18.5	1.5×10^5

HSB-2	+	13.8	24.4	81.7	1.0×10^5
	-	19.5	28.5	84.0	3.0×10^5
Molt 4	+	8.2	16.8	41.7	1.1×10^5
	-	7.6	15.3	44.4	1.7×10^5
Molt 3	+	4.0	12.9	14.0	7.5×10^3
	-	8.1	12.0	21.3	1.3×10^4

a. Cells were infected with dengue-2 virus at a m.o.i. of 5 p.f.u./cell in the presence or absence of antibody as described in Materials and Methods.

b. Anti-dengue-2 antibody was used at final dilution of 1:10,000. + represents presence of antibody and - represents absence of antibody.

C-2. Establishment of cell lines persistently infected with dengue-2 virus

We determined the ability of certain cells to become persistently infected with dengue-2 virus. K562, Raji and HSB-2 cell lines were infected with dengue-2 virus at a m.o.i. of 5 p.f.u./cell in the absence of antibody to dengue-2 virus, and were cultured for 25 weeks. Cells were resuspended at a concentration of 2×10^5/ml twice a week. The percent of dengue-2 virus antigen-positive cells was almost 100% one week after infection of K562 (Figure 8) and HSB-2 cells (Figure 9), and three weeks after infection of Raji cells (Figure 10). A high percentage (more than 70% of dengue-2 virus antigen-positive cells was observed for 25 weeks. These results demonstrate that persistent dengue-2 virus infections were readily established in myelomonocytic, B and T cell lines.

We measured dengue-2 virus titers in culture supernatant fluids 22 weeks after the infection. Infectious dengue-2 virus at the titer of 2.6×10^2 p.f.u./ml, 1.8×10^2 p.f.u./ml and 1.0×10^2 p.f.u./ml were detected in the supernatant fluids of persistently infected K562, HSB-2 and Raji, respectively. Intracellular dengue virus was then detected after three freeze-thaw cycles of 1×10^6 infected cells. Intracellular virus titers were 2.2×10^2 p.f.u./ml and 4.0×10^1 p.f.u./ml in K562 and HSB-2 cells, respectively. Intracellular virus was not detected (<10 p.f.u./ml) in persistently infected Raji cells.

Figure 8

Persistent infection of K562 cells infected with dengue-2 virus at a m.o.i. of 5 p.f.u./cell. Cells were resuspended at 2 x 10^5/ml every 3 or 4 days. Percentage of dengue-2 virus antigen-positive cells was determined using IF.

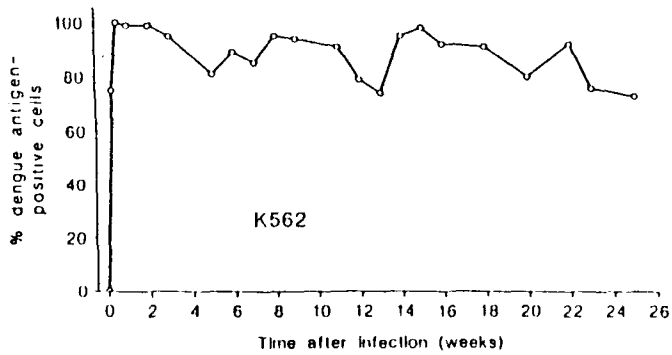


Figure 9

Persistent infection of Raji cells infected with dengue-2 virus at a m.o.i. of 5 p.f.u./cell. Cells were resuspended at 2 x 10^5/ml every 3 or 4 days. Percentage of dengue-2 virus antigen-positive cells was determined using IF.

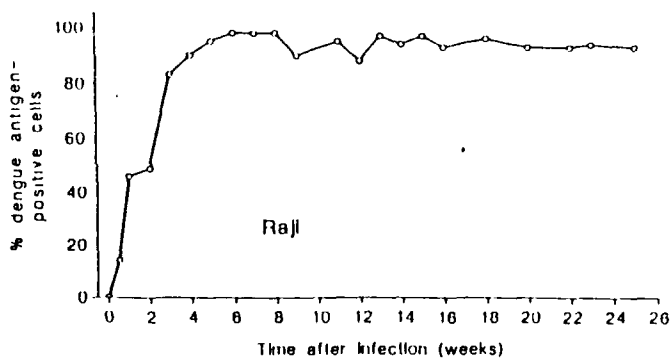
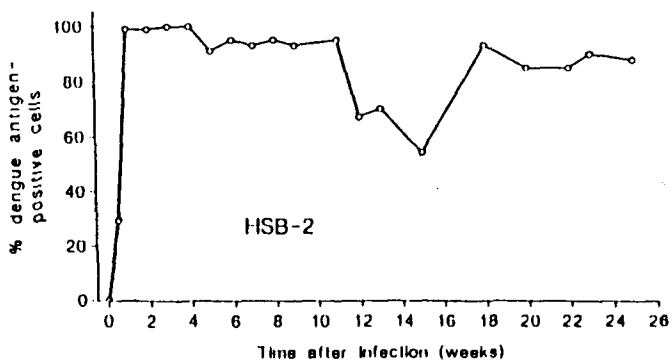


Figure 10

Persistent infection of HSB-2 cells infected with dengue-2 virus at a m.o.i. of 5 p.f.u./cell. Cells were resuspended at 2 x 10^5/ml every 3 or 4 days. Percentage of dengue-2 virus antigen-positive cells was determined using IF.



C-3. Establishment of Raji cell clones persistently infected with dengue-2 virus using a limiting dilution technique

Using a limiting dilution technique, we tried to determine whether a single dengue-2 virus antigen-positive cell can proliferate to produce multiple antigen-positive cells. Persistently infected Raji cells, 90% of which were antigen-positive, were cultured at a concentration of 0.5 cells/well. The clonal lines were examined for dengue-2 virus antigens after 3 weeks. 61 clones were established, 54 (89%) of which contained 100% antigen-positive cells, and seven (11%) of which contain no antigen-positive cells.

III. DISCUSSION

In this report we first described heterogeneity of dengue virus-specific, human $CD4^+8^-$ T cell clones. There are at least six patterns of virus- and serotype-specificities (a) dengue serotype-specific clones which recognize dengue-3 virus, but do not recognize dengue-1, -2 or -4 virus, YFV or WNV (b) dengue subcomplex-specific clones which recognize dengue-2, -3 and -4 virus, but do not recognize dengue-4 virus, YFV or WNV (c) a dengue subcomplex-specific clone which recognizes dengue-1, -2 and -3 virus but does not recognize dengue-4 virus, YFV or WNV (d) dengue serotype-crossreactive clones which recognize dengue-1, -2, -3, and -4 virus, but do not recognize YFV or WNV (e) a flavivirus-crossreactive clone which recognize dengue-1, -2, -3 and -4 virus, and WNV, but does not recognize YFV (f) flavivirus-crossreactive clones which recognize dengue-1, -2, -3 and -4 virus, YFV and WNV. HLA-restriction of cytotoxicity by dengue virus-specific $CD4^+$ T cell clones is also heterogeneous. HLA DP, DQ and DR are each used as restriction elements by individual CTL clones. Seven of the twelve examined clones recognize epitopes on the NS3 protein. Table 6 depicts summary of these results.

These dengue virus-specific $CD4^+$ T cell clones have HLA class II-restricted cytotoxic activities. We have previously reported that most of these T cell clones produce IFN after stimulation with dengue Ag (5). We have also reported that IFN , which upregulates Fc R, augments dengue virus infection of Fc R-positive monocytic cells in the presence of dengue antibodies. Based on these observations we hypothesized that $CD4^+$ T cells may contribute to the pathogenesis of DHF/DSS by producing IFN and by lysing dengue virus-infected monocytes (7,8). Epidemiological studies have shown that DHF/DSS are much more commonly observed during secondary infection with a different serotype from primary infection (1,3). Presence of serotype-crossreactive and flavivirus-crossreactive $CD4^+$ T cells supports the possibility that these T cells are activated during secondary infection with a dengue virus of an heterologous serotype and these T cells may contribute to the pathogenesis of DHF/DSS.

Seven clones recognize NS3 protein. This result is consistent with our previous observation that NS3 induced high levels of proliferation of PBMC from this donor A in bulk cultures (9). The clones which recognize NS3 are heterogeneous. They include a dengue-3-specific clone (JK37), subcomplex-specific clones (JK36 and JK46), dengue serotype-crossreactive clones (JK32, JK34 and JK39) and a flavivirus-crossreactive clone (JK43). NS3 is one of the 7 nonstructural proteins and consists of 618 amino acids (20). The amino acid homologies of NS3 are 75% between dengue-4 virus, the 814669 strain and dengue-2 virus, New Guinea C strain (21), 62% between dengue-4 virus and WNV, and 51% between dengue-4 virus and YFV (20). The presence of dengue subcomplex-specific, serotype-crossreactive and flavivirus-crossreactive clones is consistent with high levels of conservation in the amino acid sequences of NS3. HLA-restriction in lysis of target cells by these clones is also heterogeneous. These results strongly suggest that NS3 has 4 epitopes recognized by dengue virus-specific $CD4^+$ T cells. It will be important to localize these multiple T cell epitopes on NS3. This will be performed using recombinant vaccinia viruses containing truncated dengue virus NS3 genome and with synthetic peptides. Some of the CTL clones do not recognize either NS3 or NS1. Therefore, NS3 is not the only protein which contains $CD4^+$ T cell epitopes. Determination of other protein(s) which contain epitopes recognized by these T cell clones remains to be done.

In the second part of this report, we examined levels of lymphokines, monokines and soluble cell surface proteins released from activated T lymphocytes in unselected Thai children hospitalized with DHF/DSS. To determine whether differential cytokines or cellular immune responses were associated with the plasma leakage that is the pathognomonic feature of DHF/DSS, we also examined these same serum factors in patients infected with dengue virus but who had no plasma leakage (dengue fever). Children with DF were drawn from the same consecutive hospital case series as those with DHF/DSS, or they were drawn from a consecutive series of DF cases identified prospectively during a longitudinal study of illnesses leading to school absence in rural Thailand. To determine which findings in the entire set of patients with dengue infection were indicative of a host response to viral infection, we examined sera collected from healthy Thai children from the same cohort which yielded the dengue fever cases. We found evidence of marked T cell activation in patients with DHF/DSS. T cell activation in patients with DF was also present, but not as marked.

Among patients with DHF/DSS, levels of sIL-2R, sCD4, sCD8, IL-2 and IFNγ were higher than those in the sera of healthy Thai children. The levels of sIL-2R, sCD4, sCD8 and IFNγ were higher on days 1-3 than on days 7-10, and IL-2 remained at the same levels on days 1-10. It is known from <u>in vitro</u> studies that activated T lymphocytes produce IL-2 and IFNγ (11), and release

sIL-2R (14). Activated $CD8^+$ T lymphocyte release sCD8 (15,16), while activated $CD4^+$ T lymphocytes release sCD4. Therefore, our results indicate that strong activation of $CD4^+$ and $CD8^+$ T cells occurs *in vivo* in the patients with dengue who develop DHF/DSS. We previously reported that dengue virus-specific $CD4^+$ $CD8^-$ T cells are generated after dengue virus infections (6). They proliferate and produce IFNγ (5,6) and IL-2 (unpublished data) after stimulation with dengue virus antigens *in vitro*. They lyse dengue virus-infected and dengue virus antigen-pulsed target cells in an HLA class II-restricted fashion (5). We have also detected $CD8^+$ $CD4^-$ cytotoxic T lymphocytes (CTL) using lymphocytes of dengue virus-immune donors (4). $CD8^+$ T cells lyse dengue virus-infected cells in an HLA class I-restricted fashion. The high levels of sIL-2R, sCD4, sCD8, IL-2 and IFNγ reported in this paper are consistent with our observations *in vitro* and suggest that $CD4^+$ $CD8^-$ CTL and $CD4^-$ $CD8^+$ CTL are both activated *in vivo* in patients with DHF/DSS. The levels of sIL-2R, sCD4, sCD8 and IFNγ were similarly high in the patients with DHF and those with DSS. The number of patients with DHF/DSS caused by primary dengue virus infections (n=3) was too small to determine whether levels of serum factors were different from those measured in patients with DHF/DSS caused by secondary dengue virus infections (n=39).

Among patients with DF, the acute and convalescent sera contained higher levels of sIL-2R, sCD4, IL-2 and IFNγ than the sera of healthy children. SCD8 levels were not elevated in most cases; however, high levels of sCD8 were detected in the small number of patients with DF who were hospitalized. We also compared the levels of sIL-2R, sCD4, sCD8, IL-2 and IFNγ between acute sera from patients with DHF/DSS and DF. Sera of patients with DHF/DSS contained higher levels of sIL-2R, sCD4 and sCD8 than the sera of patients with DF. These results indicate that the levels of activation of $CD4^+$ and $CD8^+$ T lymphocytes are higher in DHF/DSS than in DF, and suggest that high levels of T cell activation may be associated with the pathogenesis of DHF/DSS.

Elevated levels of sIL-2R have been reported in measles (22), HTLV-1 (23) and HIV infection (24-26). Elevated levels of sCD8 have been reported in measles (22) and Epstein-Barr virus infections (16). Increased levels of IFNγ have been reported in measles (27). We also observed high levels of sIL-2R, IL-2 and IFNγ in the sera of Thai children with uncharacterized febrile diseases other than DF, DHF/DSS and Japanese encephalitis. These results suggest that T lymphocytes are activated during systemic virus infections, and that elevation of sIL-2R, sCD4, sCD8, IL-2 and IFNγ is not unique to dengue virus infections.

We did not detect elevated levels of GM-CSF, IL-1α or TNFα in patients with DHF/DSS. The normal levels of GM-CSF seem to be inconsistent with elevated levels of sIL-2, sCD4, IL-2 and IFNγ, because GM-CSF is also reported to be produced by activated T lymphocytes (12,13). This may be due to the difference in T cell

subsets which produce GM-CSF and IL-2 or IFNγ, or due to differences in the time of production and elimination. IL-1α and TNFα are produced mainly by monocytes/macrophages (17,18). These results suggest that during DHF/DSS monocytes are not releasing IL-1α or TNFα, or that monokines are rapidly eliminated from the circulation and cannot be detected by the time patients developed complications and are admitted to the hospital.

The role of cytokines in the pathogenesis of DHF/DSS is not known. IL-2 induces plasma leakage in humans at dosages above 10^5 U/kg (28,29). Although the mechanism of the plasma leakage is not clearly understood, IL-2 is known to induce lymphokine-activated killer (LAK) cells (30) and thromboxane A_2 (31), and activates endothelial cells (32), which may cause plasma leakage. Activation of the complement system, which is observed in DHF/DSS (1), was observed in patients injected with high doses of IL-2 , and the levels of plasma C3a correlated with signs of vascular leak syndrome (33). These observations suggest that the high levels of IL-2 detected in the sera of patients with DHF/DSS may be one of the factors which induce plasma leakage and shock in DSS. On the other hand, we detected similar levels of IL-2 in the sera from patients with DF, therefore, it is unlikely that IL-2 alone induces DHF/DSS.

We observed significant correlations between the severity of illness (DHF/DSS versus DF) and elevated levels of sIL-2R, sCD4 and sCD8, but it is not possible to definitively correlate the severity of illness with the levels of the lymphokines and monokines in the sera examined. Plasma leakage and hemorrhage during dengue virus infections may not be the result of any single cytokine or monokine. Marked activation of T cells does however appear to be a feature of such cases. Therefore, future examination of the interaction of immune effector cells and infected target cells will be important to determine the pathogenesis of plasma leakage and hemorrhage. It is also possible that many of the host immune responses had been already activated and some of the secreted cytokines had been eliminated or degraded before sera were obtained, because these DHF/DSS patients were admitted to the hospital after several days of illness when symptoms were severe or rapidly worsening. It would be desirable to serially evaluate a cohort of patients beginning earlier in the course of dengue virus infection to determine whether elevated serum levels of specific lymphokines or monokines predict the appearance of plasma leakage or hemorrhage.

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