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Stimulation with live dengue virus of peripheral blood mononuclear cells from a dengue 4-immune donor generated virus-specific serotype cross-reactive $CD4^-$ $CD8^+$ class I-restricted cytotoxic T lymphocytes (CTL) capable of lysing dengue virus-infected autologous fibroblasts and cells pulsed with dengue antigens of all 4 serotypes. These CTL lysed autologous fibroblasts infected with vaccinia-dengue recombinant viruses containing the E, or several non-structural dengue 4 genes. These results demonstrate that both dengue structural and non-structural proteins are targets for the HLA class I-restricted, $CD4^-$ $CD8^+$ CTL.

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HLA class II-restricted, $CD4^+$ and $CD8^-$ T cell clones which we reported previously were further characterized. The clones lysed LCL pulsed with dengue antigens and dengue virus-infected LCL. Serotype cross-reactivity in cytotoxicity is consistent with the responses observed in proliferation assay. A $CD4^+$ clone recognized a non-structural protein other than NS1 and NS2a.

To Investigate the possible role of T lymphocytes in vivo, we examined the lymphokines and soluble IL-2 receptor (SIL-2R) in the sera from DHF/DSS patients. High titers of SIL-2R, IL-2 and IFN$_\gamma$ were detected in the patients' sera. These results indicate that T lymphocytes are activated during dengue virus infections and suggest that these lymphokines may contribute to the pathogenesis of DHF/DSS.

We then investigated the possible role of IgG Fc receptor II ($Fc_\gamma RII$) in dengue virus infection. We used an erythroleukemia cell line, K562, which has $Fc_\gamma RII$, but does no have $Fc_\gamma RI$ or $Fc_\gamma RIII$, to determine if $Fc_\gamma RII$ can mediate infection by dengue virus-Ab complexes. Polyclonal mouse anti-dengue Ab significantly augments dengue virus infection of K562 cells, while normal mouse serum does not. A monoclonal Ab IV.3, which is specific for $Fc_\gamma RII$ and is known to inhibit the binding of antigen-antibody complex to $Fc_\gamma RII$, inhibits dengue Ab-mediated augmentation of dengue virus infection. It has been reported that $Fc_\gamma RII$ binds to mouse IgG1, but not to mouse IgG2a. A mouse IgG1 monoclonal anti-dengue Ab (3H5) augments dengue virus infection of K562 cells, but a mouse IgG2a monoclonal anti-dengue Ab (4G2) does not. 4G2 augments dengue virus infection of a human monocytic cell line, U937, which has $Fc_\gamma RI$. Based on these results we conclude that $Fc_\gamma RII$ mediate antibody-dependent enhancement of dengue virus infection in addition to $Fc_\gamma RI$.

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HUMAN IMMUNE RESPONSE TO DENGUE INFECTIONS

ANNUAL REPORT

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JULY 31, 1989

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FOREWORD

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

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I. INTRODUCTION

Dengue infections are a major cause of morbidity worldwide, and hemorrhagic fever and shock are very severe and frequently fatal complications of dengue infections (1). These complications are more commonly observed in individuals undergoing a secondary dengue infection with a different dengue serotype than they experienced as their primary infection (2). It has been speculated that dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are mediated by host immune mechanisms.

We have begun to define the role of dengue-specific T lymphocytes in the pathogenesis of DHF/DSS. In the previous annual report, we described the characteristics of dengue-specific, HLA class II-restricted $CD4^+$ $CD8^-$ T cell clones. Most of these clones respond to four serotypes of dengue antigens. They produced IFNγ after stimulation with dengue antigens. The IFNγ augmented dengue virus infection of human monocytes and monocytic cells in the presence of anti-dengue antibodies.

Little is known about $CD8^+$ cells in dengue infections. They do not seem to proliferate in response to soluble dengue antigens, and dengue-specific $CD8^+$ CTL have not yet been described. $CD8^+$ CTL responses have been detected to other viruses after stimulation with live virus, or with virus-infected fibroblasts. This is presumably due to a requirement for intracellular antigen processing which appears important for optimal antigen presentation in the context of class I molecules.

In this report, we describe the proliferation of peripheral blood mononuclear cells (PBMC) from a dengue 4-immune donor in response to live dengue virus, and the generation of serotype cross-reactive, $CD4^-$ $CD8^+$, HLA class I-restricted dengue-specific CTL.

II. RESULTS

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

A-1. Stimulation of dengue 4-immune PBMC with live or UV-inactivated dengue virus

PBMC taken from a dengue 4-immune donor were placed in culture with either live dengue virus or virus which had been exposed to UV light and shown to have no plaque-forming capacity. After 6 days, the cells were pulsed with ^{3}H-thymidine (^{3}H-TdR) and proliferation was assessed. The dengue 4-immune PBMC responded to dengue 4 virus (Table 1). There was also a lower but significant response to dengue 2, which indicated that the response was serotype cross-reactive. Non-immune PBMC did not respond (data

not shown), and UV-inactivated dengue 4 failed to generate a response.

Table 1. Proliferative responses of PBMC from a dengue 4-immune donor to cell-free dengue viruses[a]

	Antigens[b]	^{3}H-TdR incorporation (c.p.m.)	Stimulation index
Exp. 1	Dengue 4 virus	26712	5.6
	Dengue 2 virus	13137	2.7
	Control[d]	4789	---
Exp. 2	Dengue 4 virus	19652	16.0
	Dengue 4 virus (UV-inactivated)	627	0.9
	Control	1209	---

[a] 2×10^6 of PBMC from a dengue 4-immune donor were incubated with cell-free dengue virus at 1:3 dilution for 6 days. Cells were pulsed with 1.25 uCi ^{3}H-TdR for 8 hours before harvest.

[b] Virus titers of dengue 4 and dengue 2 viruses were 1×10^8 and 4×10^7 p.f.u./ml, respectively.

[c] The stimulation index was calculated from mean c.p.m. induced by dengue virus/mean c.p.m. induced by culture fluids of uninfected C6/36 cells.

[d] Culture fluids of uninfected C6/36 cells were used as control antigen.

A-2. Lysis of dengue virus-infected fibroblasts by dengue 4-immune PBMC stimulated with live dengue virus

PBMC from a dengue 4-immune donor were tested for cytotoxic activity against dengue virus-infected autologous fibroblasts after 7-9 days of stimulation with dengue virus. Fibroblasts were chosen as target cells to detect $CD8^+$ CTL because they do not constitutively express class II MHC antigens (3). The results in Table 2 indicate that dengue 4-immune PBMC stimulated with either dengue 2 or dengue 4 virus lysed fibroblasts infected with either serotype. Uninfected cells were also lysed, but the levels of lysis were always lower than those against virus-infected targets. These results show that the cytotoxic response is serotype cross-reactive.

Table 2. Lysis of dengue virus-infected fibroblasts by dengue 4-immune PBMC stimulated with dengue viruses[a]

Exp.	PBL stimulated with	% specific ^{51}Cr release from fibroblasts infected with: dengue 4	dengue 2	Mock
1.	Dengue 4 virus	26	26	2
	Dengue 2 virus	28	24	7
	Mock	2	5	2
2.	Dengue 4 virus	62	58	2

[a]In exp. 1 the effector to target ratio was 40:1 in a 4 hour assay. In exp. 2 the effector to target ratio was 100:1 in a 4 hour assay.

A-3. HLA class I-restriction of the lysis of dengue virus-infected fibroblasts

We examined whether the lysis of dengue virus-infected fibroblasts by CTL is HLA class I-restricted, using monoclonal antibodies to HLA class I and HLA-DR. Inclusion in the cytotoxicity assay of an antibody against class I antigens, W6/32, inhibited the killing of dengue 2-infected fibroblasts by 48%, which indicates that much of the killing was class I antigen-restricted (Table 3). An antibody to class II DR antigens, OKIa-1, inhibited the killing poorly, showing that the killing was not DR-restricted. Since fibroblasts do not express any class II antigens under these conditions (3), killing restricted by the other class II (DP, DQ) can also be excluded.

Table 3. Inhibition of the lysis of dengue-infected fibroblasts by monoclonal anti-HLA class I antibody, W6/32[a]

Monoclonal Ab added to assay	% specific ^{51}Cr release	% inhibition
None	65	--
W6/32	34	48
OKIa1	55	15

[a]PBL from dengue 4-immune donor was incubated with dengue 4 virus for 8 days, and CTL activity was examined on dengue 2-infected fibroblasts. Effector target cell ratio was 100:1 in a 4 hour assay.

A-4. Phenotype of the CTL generated in bulk culture

Treatment of the effector population with anti-CD8 Ab and C′ resulted in the death of 37% of these cells, and reduced their lytic capacity against dengue 2-infected fibroblasts by 61% (Table 4). Anti-CD4 Ab and C′ treatment similarly killed 35% of the effector population, but failed to inhibit the cytotoxic activity (Table 4). Anti-Leu 11b Ab and C′ treatment failed to induce either a significant decrease in viability (data not shown) or to reduce the cytotoxic capacity of effector cells (Table 4). Taken together, these results indicate that the effector cells have $CD4^-$ $CD8^+$ phenotype.

Table 4. Depletion of CTL activity by the treatment with anti-CD8 antibody and complement[a]

Treatment of target cells with complement and	% specific ^{51}Cr release	% inhibition
---	46	--
Anti-CD4	50	0
Anti-CD8	18	61
Anti-CD16	44	4

[a]Effector cells were treated with monoclonal antibodies and complement. Effector:target ratio was 100:1 in a 4 hour assay.

A-5. Cross-reactive lysis of dengue antigen-pulsed lymphoblastoid cell line (LCL) and fibroblasts

It is difficult to infect an adequate percentage of human LCL and fibroblasts in vitro with dengue virus types 1 and 3; therefore, we pulsed fibroblasts and LCL with dengue antigens prepared from dengue virus-infected, glutaraldehyde-fixed Vero cells and used them as targets for dengue virus-stimulated PBMC. Table 5 shows that fibroblasts and LCL pulsed with dengue antigens were efficiently lysed by dengue 4 virus-stimulated PBMC, but lysis of control antigen-treated or untreated fibroblasts was minimal. Thus these CTL are cross-reactive against all 4 dengue serotypes. We characterized the MHC restriction of killing against LCL by including antibodies to HLA class I (W6/32) and HLA class II (OKIa-1) antigens in the cytotoxicity assays. W6/32 inhibited the killing by 57% whereas OKIa-1 had no effect. These data and the fact that fibroblasts, which lack class II antigens, are killed argue strongly that this lysis is mediated by class I-restricted CTL.

Table 5. Lysis of dengue antigen-pulsed LCL and fibroblasts by HLA class I-restricted CTL[a]

Exp.	Target cell type	% specific release of target cells treated with: dengue 1	dengue 2	dengue 3	dengue 4	control
1.	fibroblasts	21	56	27	19	5
2.	fibroblasts	ND	ND	ND	36	5
3.	LCL	ND	ND	ND	52	7

[a]Effectors were dengue 4-immune PBMC stimulated for 8 days with dengue 4 virus. The effector to target ratio was 100:1 and the assay length was 6 hours. Target cells pulsed with dengue antigens or control antigens were used as targets the following day.

A-6. Lysis of fibroblasts infected with dengue-vaccinia recombinants

We examined the proteins which are targets for dengue-specific class I-restricted CTL by infecting fibroblasts with dengue-vaccinia recombinant viruses, each of which contains a different portion of the dengue 4 genome. Recombinant A contains genes which code for dengue proteins NS1, NS2a, NS2b, NS3, NS4a, and 84% of NS4b. Recombinant B codes for C, pre-M, E, NS1 and NS2a. Recombinant C includes genes coding for NS1 and NS2a, and recombinant E codes for the E protein. Recombinant D is the parental vaccinia virus which contains the lac Z gene and has none of the dengue genome and serves as a control. Table 6 shows that CTL lysed fibroblasts infected with recombinants A, B and E, but not C and D. These results show that E is a target protein for these CTL, but NS1 and NS2a are not. The data also imply that at least one of the NS2b, NS3, NS4a, and NS4b proteins is a target for these CTL. From these studies, we cannot determine whether C, Pre-M, and NS5 contain target epitopes for CTL. Recombinant vaccinia viruses which contained the genes for the dengue 2 C, pre-M, and E proteins and the genes for dengue 2 NS1, NS2a proteins were also used to infect autologous fibroblasts. Consistent with the concept that these CTL are cross-reactive, fibroblasts infected with the dengue 2 recombinant expressing C, pre-M, and E proteins were lysed by dengue 4 virus-stimulated, dengue 4-immune PBMC. However, these PBMC did not lyse fibroblasts infected with the dengue 2 recombinant expressing NS1 and NS2a (data not shown).

Table 6. Lysis of fibroblasts infected with dengue-vaccinia recombinants[a]

Fibroblasts infected with:	% specific ^{51}Cr release: Exp. 1	Exp. 2
dengue 2	94	ND
dengue 4	72	31
A=v[NS1, NS2a, NS2b, NS3, NS4a, NS4b]	39	17
B=v[C, pre-M, E, NS2, NS2a]	8	13
C=v[NS1, NS2a]	-2	0
D=vaccinia control	-	-
E=v[E]	13	28
Mock-infected	-4	-1

[a]Effects were from dengue 4-immune PBMC stimulated for 8 days with dengue 4 virus. Assay length was 4 hours. E:T was 100:1 in Exp. 1 and 40:1 in Exp. 2. Lysis against targets infected with vaccinia virus (D=vaccinia control) was 12% in both experiments, which was subtracted for clarity.

B. Dengue-specific, HLA class II-restricted CTL clones

In the previous annual report we described the establishment and characterization of dengue-specific HLA class II-restricted CTL clones. We analyzed clones of dengue serotype cross-reactive T lymphocytes derived from the peripheral blood mononuclear cells (PBMC) of a donor who had been infected with dengue 3 virus. These PBMC responded best to dengue 3 antigen, but also responded to dengue 1, 2 and 4 antigens, in bulk culture proliferation assays. Twelve dengue antigen-specific clones were established using a limiting dilution technique. All of the clones had $CD3^+$, $CD4^+$, $CD8^-$ phenotypes. Eight clones responded to dengue 1, 2, 3 and 4 antigens and are cross-reactive, while four other clones responded predominantly to dengue 3 antigen. These results indicate that the serotype cross-reactive dengue-specific T lymphocyte proliferation observed in bulk cultures reflects the cross-reactive responses detected at the clonal level. Serotype cross-reactive clones produced high titers of interferon gamma (IFNγ) after stimulation with dengue 3 antigens, and also produced IFNγ to lower levels after stimulation with dengue 1, 2 and 4 antigens. We have further characterized these T cell clones.

B-1. Cytotoxic activities of dengue specific T cell clones to autologous LCL pulsed with dengue antigens

Two serotype cross-reactive clones JK34 and JK36 were examined for cytotoxic activity against autologous LCL pulsed with dengue and yellow fever antigens. They lysed dengue 3 antigen-pulsed LCL and also lysed LCL pulsed with dengue antigens of other serotypes (Table 7). JK36 which did not proliferate after stimulation with dengue 1 antigen did not lyse dengue 1 antigen-pulsed LCL. A serotype-specific clone JK37 lysed dengue 3 antigen-pulsed LCL, but did not lyse the other target cells. These results indicate that dengue-specific clones have dengue antigen-specific cytotoxic activities and serotype cross-reactivity in cytotoxicity is consistent with the responses observed in proliferation assays.

Table 7. Cytotoxic activities of dengue specific T cell clones to autologous LCL pulsed with dengue antigens[a]

Autologous LCL pulsed with Ag	% specific 51Cr release		
	JK34	JK36	JK37
Dengue 1	24	0	1
Dengue 2	51	42	0
Dengue 3	52	43	18
Dengue 4	43	24	0
Yellow fever	0	1	0
Control	0	0	0
None	0	0	0

[a] 2.5×10^3 target cells were incubated with effector cells for 4 hours. Effector: target ratio was 6:1 for JK34, 5:1 for JK36, and 2:1 for JK37.

B-2. Lysis of dengue 2 virus-infected autologous LCL by dengue serotype cross-reactive T cell clones

We examined dengue-specific clones for their cytotoxic activities to dengue 2 virus-infected autologous LCL. All the cross-reactive clones but one (JK27) lysed dengue 2 virus-infected autologous LCL (Table 8). These clones did not lyse uninfected LCL or K562 cells. Dengue 3 serotype-specific clones did not lyse dengue 2-infected LCL, uninfected LCL or K562. These results suggest that serotype cross-reactive T cells may lyse dengue-infected cells during secondary infections.

Table 8. Lysis of dengue 2 virus-infected autologous LCL by serotype cross-reactive T cell clones[a]

Cones	Effector: target ratio	% specific ^{51}Cr release: Dengue 2-infected autologous LCL	Uninfected autologous LCL	K562
Serotype cross-reactive				
JK26	2	71	0	0
JK27	2	0	0	0
JK28	2	85	0	0
JK32	2	42	2	0
JK33	2	16	0	1
JK34	2	43	0	0
JK35	2	35	0	1
JK36	2	59	0	0
Serotype specific				
JK30	3	0	0	0
JK31	3	0	0	0
JK37	4	2	2	0

[a] 2.5×10^3 target cells were incubated with effector cells for 4 hours. % specific ^{51}Cr release was calculated by the formula described in Materials and Methods.

B-3. HLA class II Ag-restricted lysis of target cells by T cell clones

HLA restrictions of the lysis of target cells by dengue-specific T cell clones were examined using monoclonal antibodies to HLA antigens. Monoclonal anti-HLA DP antibody, B7/21.7, inhibited the lysis of dengue 3 Ag-pulsed autologous LCL and dengue 2 virus-infected autologous LCL by JK34 and JK35. Monoclonal anti-HLA DQ antibody, S3/4, inhibited the lysis of these target cells by JK36 (Table 8). Monoclonal anti-HLA class I antibody, W6/32, did not inhibit the lysis of target cells by these T cell clones (data not presented). These results indicate that dengue-specific, $CD4^+$ T cell clones lyse target cells in an HLA class II-restricted fashion, and that HLA DP or DQ antigens are the restricting antigens for the clones examined.

Table 9. HLA class II Ag-restricted lysis of target cells by dengue-specific T cell clones[a]

Target cells	Monoclonal antibodies to[b]	% specific 51Cr release JK34	JK35	JK36
Dengue 3 Ag-pulsed autologous LCL	--	73	22	83
	HLA DR	77	30	72
	HLA DP	5	2	70
	HLA DQ	79	27	9
Dengue 2-virus infected autologous LCL	--	57	22	44
	HLA DR	57	19	41
	HLA DP	8	2	50
	HLA DQ	59	22	8

[a] 2.5×10^3 target cells were incubated with 1.25×10^4 effector cells for 4 hours in the presence of monoclonal antibodies, at final dilution of 1:80.

[b] OKIa1, B7/21.7 and S3/4 were used as anti-HLA DR, anti-HLA DP and anti-HLA DQ antibodies, respectively.

B-4. Determination of dengue proteins which contain epitope recognized by CTL clones

We have begun to determine dengue proteins which contain epitopes recognized by dengue-specific, HLA class II restricted CTL clones. Autologous lymphoblastoid cell lines (LCL) infected with recombinant vaccinia virus containing dengue genome were used as target cells. A dengue-specific, HLA class II-restricted clone JK34 lysed LCL expressing dengue proteins NS1, 2a, 2b, 3, 4a, and 4b, but did not lyse LCL expressing C, Pre-M, E, NS1, and NS2a, LCL expressing E or those expressing NS1 and NS2a (Table 10).

Table 10. Lysis by JK34 of LCL infected with recombinant vaccinia virus containing dengue genomes[a]

LCL infected with	% specific 51Cr release
V [C, Pre-M, E, NS1, NS2a][b]	0
V [NS1, NS2a, NS2b, NS3, NS4a, NS4b]	32
V [E]	4
V [NS1, NS2a]	0
Vaccinia Control	0
None	6
LCL coated with D4 antigen	40

[a]JK34 is a HLA class II-restricted CTL clone. Effector:target ratio was 18:1. 4 hour assay.
[b]Vaccinia virus which contains dengue genome coding for dengue proteins shown in the parenthesis.

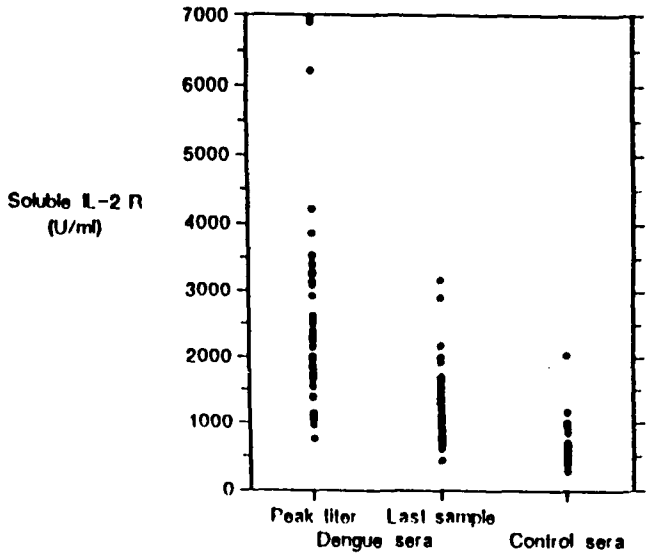
C. Detection of lymphokines, cytokines and lymphocyte surface protein in the sera from dengue infected patients

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 tried to examine the levels of various cytokines and lymphocyte surface protein in the sera from dengue-infected patients. We hypothesize that the levels of these lymphokines and soluble interleukin 2 receptors (SIL-2R) reflect the immune responses to dengue viruses in vivo, and that some of the lymphokines may cause severe complications of dengue virus infections. These DHF/DSS patients were serially bled starting the day of the admission to the hospital.

C-1. Detection of SIL-2R in the sera from DHF/DSS patients

High titers of SIL-2R were detected in the sera from DHF/DSS patients. The titer decreased by days 7-9 after admission, but was still higher than the levels detected in the sera from healthy Thai children (Figure 1).

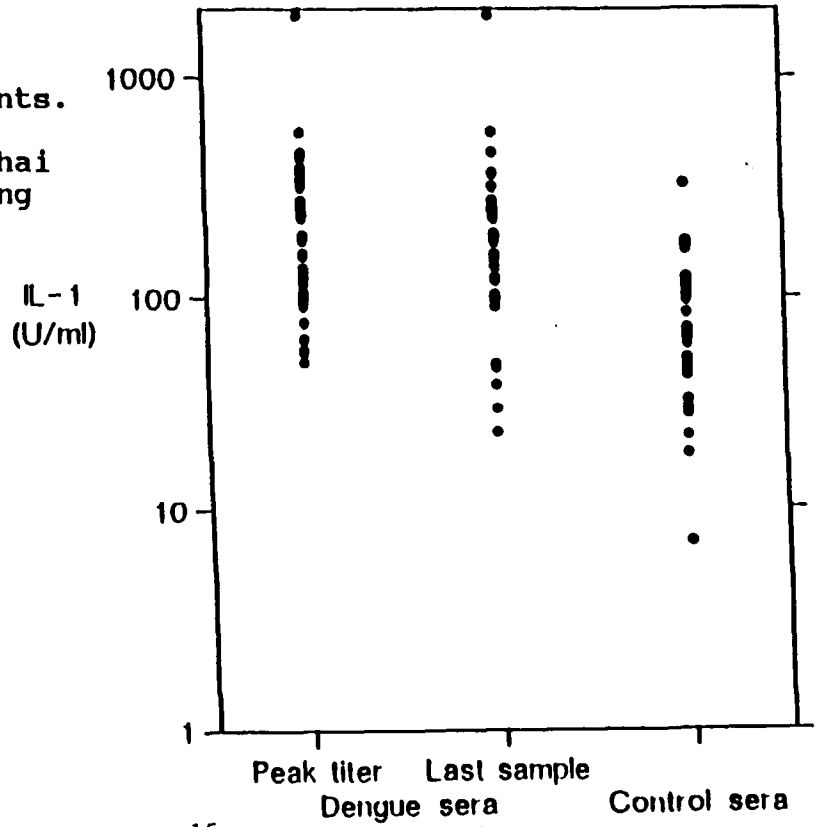
Figure 1. Soluble IL-2R in the sera from dengue patients. SIl-2R in the sera from patients and healthy Thai donors was assayed using ELISA.



C-2. Detection of interleukin 1 α (IL-1α) in the sera from DHF/DSS patients

Serum IL-1 was detected in the sera from DHF/DSS patients and in the sera from normal Thai children. The titers in the DHF/DSS sera were not different from those in the control sera (Figure 2).

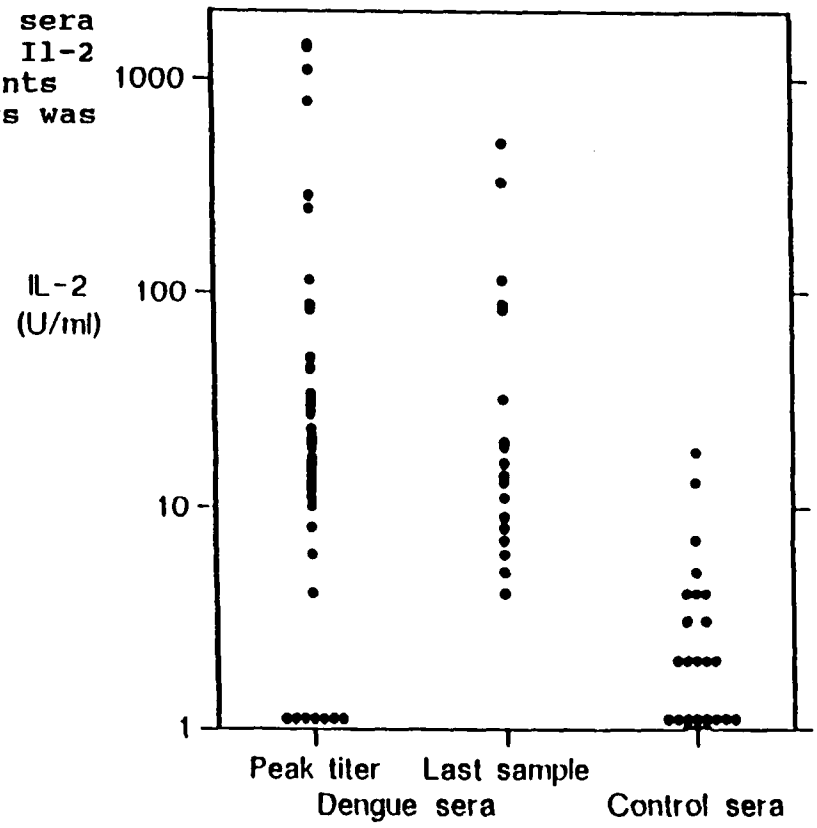
Figure 2. IL-1 in the sera from dengue patients. Il-1 in the sera from patients and healthy Thai donors was assayed using ELISA.



C-3. Interleukin 2 (IL-2) in the sera from dengue patients

Serum IL-2 level in the control Thai sera ranges from 0-18 u/ml. In the sera from DHF/DSS patients, the titer of IL-2 was much higher and ranges from 0-1424 u/ml. The titer stays at high levels at days 6-7 after admission (Figure 3).

Figure 3. Il-2 in the sera from dengue patients. Il-2 in the sera from patients and healthy Thai donors was assayed using ELISA.



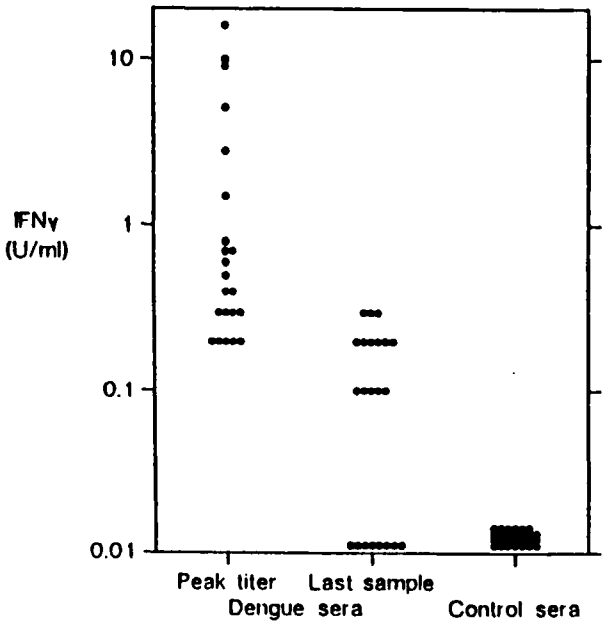
C-4. Tumor necrosis factor α (TNF-α) in the sera from dengue patients

We have examined 40 sera from DHF/DSS patients for TNF- , but TNF- was not detected in any sera (data not presented).

C-5. Interferon gamma (IFNγ) in the sera from dengue patients

We detected IFN (0.2-16 u/ml) in the sera from dengue patients. IFNγ was still detected in most of the last samples (day 7) ranging from 0-0.6 u/ml. IFNγ was not detected in the sera from healthy Thai children (Figure 4).

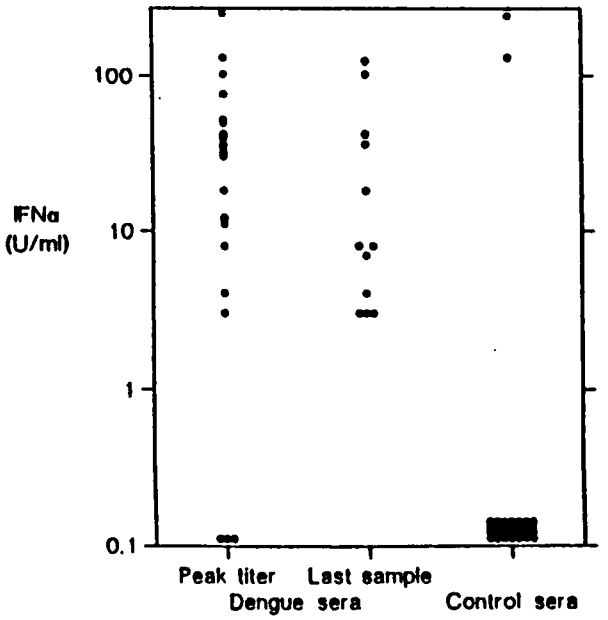
Figure 4. IFNγ in the sera from dengue patients. IFNγ in the sera from patients and healthy Thai donors was assayed using RIA.



C-6. Interferon alpha (IFNα) in the sera from dengue patients

We detected IFNα (0-250 u/ml) in the sera from dengue patients. IFN was still detected in most of the last samples, ranging 0-124 u/ml. IFNα was not detected in the sera from most of the healthy Thai children (Figure 5).

Figure 5. IFNα in the sera from dengue patients, IFN in the sera from patients and healthy Thai donors was assayed using RIA.



D. IgG Fc receptor II (FcγRII)-mediated enhancement of dengue virus infection

IgG Fc receptor II (FcγRII) is a 40 kilodalton (kd) molecule detected on monocytes, neutrophils, eosinophils, platelets and B cells (4,5). The functions of FcγRII have been defined in comparison with two other IgG Fc receptors, FcγRI and FCγRIII. FcγRI is a 72 kd molecule that is detected exclusively on monocytes and IFNγ-treated granulocytes (4,6). FcγRIII is a 50-70 kd molecule that is detected on neutrophils, eosinophils, macrophages, and natural killer cells (4). FcγRII are cytotoxic trigger molecules as are FcγRI and FcγRIII (7,8). Cross linking of FcγRII by specific antibodies induces superoxide as does the cross linking of FcγRI (9). FcγRII on monocytes support mouse IgG1 anti-CD3-induced T cell activation, while FcγRI support mouse IgG2a anti-CD3-induced T cell activation (10). These reports suggest that biological functions of FcγRII may be similar to those of FcγRI. In the previous annual report, we reported that IFNγ further augments dengue virus-infection of human monocytic cells by increasing the number of Fc RI detected by monoclonal Ab 32. Therefore, FcγRI can mediate antibody-dependent enhancement (ADE) of dengue virus infection. In this report we analyzed whether FcγRII can mediate ADE of dengue-virus infections. FcγRII have a much broader range of cellular expression than FcγRI (4), and are modulated by colony stimulating factor which does not modulate FcγRI (11). Therefore, it is important to elucidate the role of FcγRII in dengue virus infections in order to better understand dengue virus-human cell interactions and the pathogenesis of dengue virus infections. To address this question, we used an erythroleukemia cell line, K562, which has FcγRII but does not have FcγRI or FcγRIII, and an FcRII-specific monoclonal Ab, IV.3, which is known to inhibit the binding of antigen-antibody complex to FcγRII (9,10).

D-1. Detection of FcγRII but not FcγRI or FcγIII on K562

K562 cells were examined for the expression of FcγRI, FcγRII and FcγRIII by quantitative flow cytometry using specific monoclonal antibodies (mAb) 32, IV.3 and anti-Leu11a, respectively. K562 expressed high levels of FcγRII, but did not express FcγRI (Figure 6) or FcγRIII (data not presented). U937 expressed FcγRI and FcγRII (Figure 6), but did not express FcγRIII (data not presented). These results are consistent with previous reports by other investigators (11). The K562 cell line was selected for defining the role of FcγRII in dengue virus infections because it expresses only FcγRII.

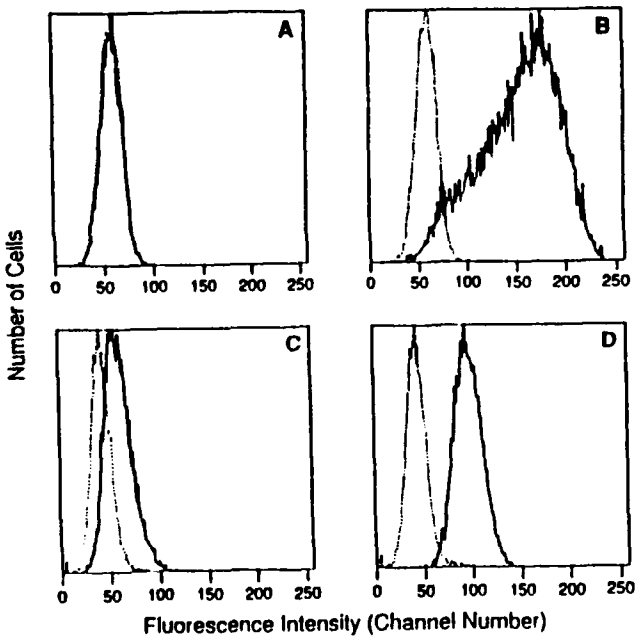


Figure 6: Indirect immunofluoroscence analysis of FcγRI and FcγRII on K562 and U937 cells. K562 and U937 were stained with RcγRI-specific mAb 32 and FcγRII-specific mAb IV.3 as described in Materials and Methods. Dotted line represents staining with control mouse IgG1 and solid line represents staining with mAb 32 and IV.3. A, K562 stained with 32; B, K562 stained with IV.3; C, U937 stained with 32; D, U937 stained with IV.3.

D-2. Augmentation of dengue virus infection of K562 by anti-dengue antibody

K562 cells were infected with dengue virus in the presence or absence of anti-dengue 2 mouse sera at a final dilution of $1:10^4$, and the percentage of dengue antigen-positive cells were examined 24 and 48 hours after infection. A dose response study showed that anti-dengue 2 serum enhanced infection best when used at a final dilution of $1:10^4$ (data not presented). K562 cells which were infected with dengue virus in the presence of the diluted polyclonal preparation of anti-dengue antibody contained a significantly higher percentage of antigen-positive cells than those infected in the absence of antibody (Table 11). Normal mouse serum, which does not contain anti-dengue antibodies, did not enhance infection at any dilutions ranging from 1:10 to $1:10^7$. These results suggested that anti-dengue antibodies enhance dengue virus infection by binding to FcγRII after the formation of virus-antibody complexes.

Table 11. Augmentation of dengue virus infection of K562 cells by anti-dengue antibody[a]

Exp.	m.o.i. (p.f.u./ cell)	Incubation time (hours)	% of dengue antigen-positive cells[b] With anti-dengue antibody	No antibody
1	0.125	24	26.3[c]	18.0
2	0.125	24	18.4[d]	12.7
3	5	24	90.8[e]	57.8
4	0.03	48	12.8[f]	5.6
5	0.125	48	20.8[g]	11.0

a. K562 cells were infected with dengue virus in the presence or absence of anti-dengue 2 mouse sera at a final dilution of $1:10^4$. The percentage of dengue antigen-positive cells were determined by FA staining.
b. The percentage of antigen-positive cells was compared between the cells infected in the presence of anti-dengue antibody and those infected in the absence of antibody by Chi square test.
c. $p<0.05$
d. $p<0.05$
e. $p<0.001$
f. $p<0.02$
g. $p<0.01$

D-3. Inhibition of anti-dengue antibody-mediated infection by monoclonal antibody specific for FcγRII

MAb IV.3, which is specific for FcγRII and is known to inhibit the binding of antigen-antibody complexes to FcγRII, was used to prove that enhancement of dengue virus infection by anti-dengue antibody is FcγRII-mediated. K562 cells were incubated with mAb IV.3 and were then infected with dengue virus in the presence or absence of anti-dengue antibody. MAb IV.3 inhibited infection when cells were infected in the presence of anti-dengue antibody, but not when cells were infected in the absence of antibody. MAb 32, which is specific for FcγRI, did not inhibit infection in the presence or absence of anti-dengue antibody (Table 12, Expt. 1). The Fab fragment of mAb IV.3 was then used to pretreat K562 cells prior to infection with dengue virus. The Fab of IV.3 also inhibited infection by virus-antibody complex, but did not inhibit infection by dengue virus alone (Table 12, Expt. 2); whereas, the $F(ab')_2$ of 32 did not affect infection. These results indicate that enhancement of infection by anti-dengue antibody is FcγRII-mediated.

Table 12. Anti-FcγRII monoclonal antibody inhibits antibody-mediated enhancement of dengue virus infection of K562 cells[a]

	Anti-FcγR antibodies	% dengue antigen-positive cells with anti-dengue antibody	% dengue antigen-positive cells no antibody
Exp. 1	None	40.6[d]	31.5[d]
	IV.3	21.2[b]	31.3[c]
	32.2	40.9[c]	32.3[c]
Exp. 2	None	28.6[e]	21.8[e]
	IV.3 (Fab)	16.7[b]	17.7[c]
	32.2 ($F(ab')_2$)	25.5[c]	21.7[c]

a. K562 cells were incubated with monoclonal Ab IV.3 or 32 at a concentration of 100 ug/ml at 4°C for 1 hour. Cells were washed once and infected with dengue virus or dengue virus-antibody complex at a m.o.i. of 0.125 p.f.u./cell. Percentage of dengue antigen-positive cells was examined using FA staining 24 hours after infection.
b. $P<0.001$ compared with the cells not pretreated with anti-FcR antibodies by Chi square test.
c. $P>0.2$ (not significant) compared with the cells not pretreated with anti-FcR antibodies by Chi square test.
d. The percentage of dengue antigen-positive cells was compared by Chi square test between the cells infected in the presence of anti-dengue antibody and those infected in the absence of anti-dengue antibody ($p<0.01$).
e. Same as d ($p<0.05$).

D-4. Enhancement of dengue virus infection of K562 cells by mouse IgG1 monoclonal anti-dengue antibody, but not by mouse IgG2a monoclonal antibody

It has been reported that FcγRII bind to mouse IgG1 but not to mouse IgG2a, and that FcγRI bind to mouse IgG2a and not to mouse IgG1 (4). We used a mouse IgG1 monoclonal anti-dengue antibody (3H5) and a mouse IgG2a monoclonal anti-dengue antibody (4G2), to examine whether the mouse IgG1 antibody enhances dengue virus infection of FcγRII-positive K562 cells. MAb 3H5 enhanced dengue virus infection at final dilutions of $1:10^3$ to $1:10^6$ and MAb 4G2 did not enhance infection of K562 cells (Figure 7). These results are consistent with those shown in Figure 1 and Table 2, and suggest that FcγRII mediates antibody-dependent enhancement of dengue virus infection. 4G2, which did not enhance dengue virus infection of K562 cells, enhanced infection of U937 cells which have FcγRI (Table 13). MAb IV.3 did not inhibit enhanced infection by 4G2. These results confirmed our previous report that FcγRI on U937 cells can mediate ADE of dengue virus infection (12).

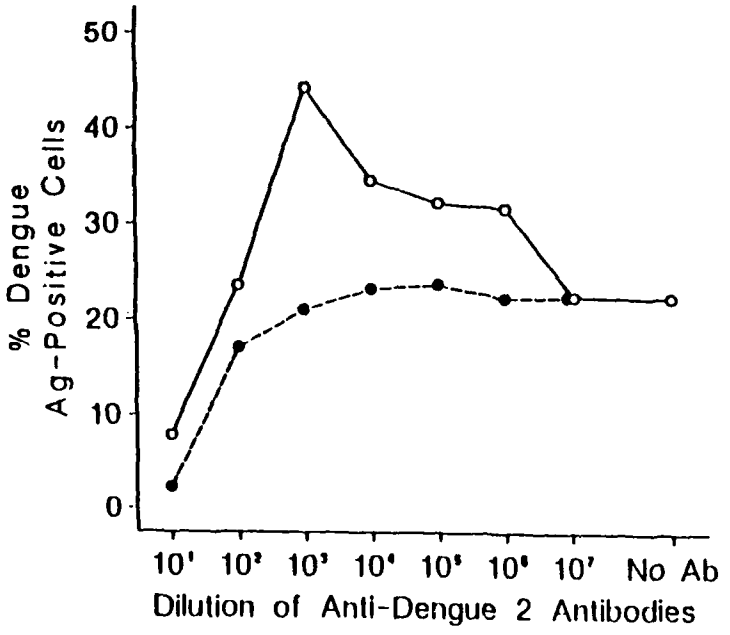


Figure 7. Enhancement of dengue virus infection of K562 cells by mouse IgG1 monoclonal anti-dengue antibody (3H5). K562 cells were infected with dengue 2 virus at a m.o.i. of 0.125 p.f.u./cell in the presence of mouse IgG1 monoclonal anti-dengue antibody (3H5) (o) and mouse IgG2a monoclonal anti-dengue antibody (4G2) (●). Percentage of dengue antigen-positive cells was compared by Chi square test between the cells infected in the absence of anti-dengue Ab and those infected in the presence of 3H5. $P<0.001$ at $1:10^3$; $p<0.01$ at $1:10^4$; $p<0.02$ at $1:10^5$; $p<0.05$ at $1:10^6$.

Table 13. Mouse IgG2a anti-dengue antibody enhances dengue virus infection of U937 cells[a]

	% dengue antigen-positive cells			
Anti-Fc RII	No	Mouse IgG2a anti-dengue Ab(4G2)		
Antibody	anti-dengue Ab	$1:10^3$	$1:10^4$	$1:10^5$
None	2.0	9.0[c]	36.9[c]	6.0[d]
IV.3	2.0	5.2[e]	38.1[c]	6.4[d]

a. U937 cells were infected with dengue 2 virus at a m.o.i. of 5 p.f.u./cell in the presence of 4G2 antibody at various dilutions. Percentage of dengue antigen-positive cells were examined using FA staining 24 hours after infection.
b. The percentage of dengue antigen-positive cells was compared by Chi square test between the cells infected in the absence of anti-dengue Ab and those infected in the presence of anti-dengue Ab.
c. $p<0.001$
d. $p<0.01$
e. $p<0.05$

III. DISCUSSION

In this report we first described serotype cross-reactive $CD8^+$ $CD4^-$ class I-restricted dengue-specific CTL obtained after stimulation with live virus of PBMC from a dengue-immune individual. The evidence that lysis was mediated by class I-restricted $CD8^+$ CTL is the following: 1) antibody and C' treatment of the effectors using anti-CD8 Ab or anti-CD3 Ab but not anti-CD16 Ab or anti-CD4 Ab depleted the killing (Table 2); 2) antibody to class I but not class II antigens decreased the level of lysis (Table 3); 3) virus-infected fibroblasts, which do not express MHC class II antigens (3), were specifically lysed (Tables 2, 5 and 6). In other viral systems (13,14), the use of live virus or virus-infected fibroblasts has also been necessary to induce class I-restricted $CD8^+$ CTL. This may be due to the requirement for intracellular antigen synthesis sometimes required for optimal presentation of antigen in the context of class I molecules (15-17). Stimulation of PBMC with soluble dengue antigens generated $CD4^+$ class II-restricted CTL. We failed to induce $CD8^+$ CTL with soluble dengue antigens despite inducing good levels of proliferation. However, fibroblasts and LCL pulsed with these antigens were sensitive to class I-restricted CTL-mediated lysis. This appears to be consistent with a report which showed that influenza virus proteins could bind to class I antigens displayed on the plasma membrane, and these antigen-pulsed targets were sensitive to lysis by influenza-specific $CD8^+$ class I-restricted CTL (18).

A rather large amount of dengue virus was necessary for optimal CTL induction (10^7-10^8 p.f.u.). During secondary dengue infections, the increased amount of virus present due to antibody-mediated enhancement of infection in Fc-receptor positive cells and the presence of memory CTL may result in a more vigorous CTL response than is usually present during primary infections. This CTL response may contribute to both the enhanced destruction of virus-infected cells and to immunopathology. Although DHF/DSS are more commonly observed during secondary dengue infections, some infants 6-12 months of age with maternally-derived enhancing antibodies develop DHF/DSS during primary dengue infections (2). This may be triggered by a primary CTL response against an augmented number of infected monocytes.

In order to postulate that CTL play a role in viral clearance and DHF/DSS during secondary dengue infections, they must be serotype cross-reactive with regard to specificity for induction and lysis. Stimulation by dengue 2 virus of PBMC from a dengue 4-immune individual inducted proliferation and CTL capable of lysing dengue 2-infected targets (Tables 1 and 2). Stimulation of these PBMC with dengue 4 virus resulted in CTL capable of lysing target cells expressing all 4 serotypes of dengue virus (Table 5), thus demonstrating the cross-reactive nature of these CTL.

In the second part of this report, we further characterized dengue-specific, HLA class II-restricted, $CD4^+$ $CD8^-$ T cell clones which we had described in the previous annual report. Cytotoxic functions of these HLA class II-restricted T cell clones were examined using dengue 2 virus-infected autologous LCL because epidemiological studies in Thailand have shown that secondary infections with dengue 2 virus induced higher rates of DHF/DSS than did secondary infections with the other serotypes of dengue virus (19). All but one serotype cross-reactive clones lysed dengue 2-infected autologous LCL. These clones did not lyse uninfected LCL or K562 cells. The lysis of dengue-infected cells by the clones examined was inhibited by anti-HLA-DP and anti-DQ antibodies; therefore, these serotype cross-reactive cytotoxic T cell clones are HLA class II-restricted. It is known that monocytes are the cells which best support dengue virus infection (20), and monocytic cells with dengue antigens have been observed in DHF/DSS patients (21,22). It has been hypothesized that lysis of dengue-infected monocytes may lead to DHF/DSS (1). Therefore, it is important to learn whether these serotype cross-reactive CTL clones can lyse dengue 2 virus-infected autologous monocytes. Two of the serotype cross-reactive clones we have examined to date lysed dengue type 2 virus-infected autologous monocytes, but they did not lyse uninfected monocytes (data not presented).

Currently there is no effective vaccine against dengue. In order to design a safe and effective vaccine and to understand the pathogenesis of DSS, it is important to learn which dengue virus-coded proteins induce protective T cell responses and which responses may lead to DHF/DSS. Our studies using dengue 4-vaccinia constructs indicate that E protein and one or more of the NS2b, NS3, NS4a, and NS4b proteins are targets for $CD8^+$ CTL. Some of the HLA class II-restricted, $CD4^+$ CTL clone recognize epitope(s) on NS proteins other than NS1, NS2a and NS5. Since E protein also contains epitopes for cross-reactive antibodies which can mediate immune enhancement (23), there is concern about using it in a vaccine. NS proteins are not expressed in virions, and may be candidates for use in a subunit vaccine. It is therefore important to elucidate which NS proteins are recognized by cross-reactive T cells and which NS proteins are targets for CTL-mediated clearance and/or CTL-mediated DHF/DSS.

NS1 or NS2a do not seem to contain CTL epitopes in this system, despite the fact that NS1 is a highly conserved protein among all 4 dengue serotypes, and it induces a serotype cross-reactive antibody response (24). Immunization of mice with NS1 protein protects mice against intracerebral challenge with dengue virus, but the protection is not serotype cross-reactive (24). Recently, two of us reported that immunization with a cell lysate containing dengue proteins C, pre-M, E, NS-1 and NS2a protects mice against dengue virus encephalitis (25). Mice immunized with this preparation develop antibody responses to NS1 but lack consistently detectable virus neutralizing antibodies. Mice immunized with a dengue-vaccinia recombinant containing the gene

for dengue 4 E protein also resisted a fatal challenge with dengue 4 virus; anti-dengue antibody titers were either low or undetectable in these mice (26). Cell-mediated immune responses were not analyzed in those experiments, and the mechanism of protection remains speculative.

Further studies are needed to better define the proteins and epitopes which are recognized by dengue-specific CTL and their MHC haplotype restrictions. This information could be important in determining which elements of the CTL response contribute to recovery from dengue and to the severe complications of dengue infections.

In order to understand the immune responses to dengue viruses in vivo, we examined cytokines and T cell surface proteins in the sera from dengue-infected patients. We detected high titer of IL-2, IFNγ, IFNα and SIL-2R in the sera from DHF/DSS patients. It has been reported that IL-2 and IFNγ are produced by activated T lymphocytes, and SIL-2R is released from activated T lymphocytes. Therefore, these results indicate that dengue-specific T lymphocytes are activated in vivo and suggest that dengue-specific T lymphocytes may contribute to the pathogenesis of DHF/DSS by producing lymphokines. It has been reported that IL-2 can induce plasma leakage and pulmonary edema (27) which can be observed in DHF/DSS patients. IFNγ can augment dengue virus infection of Fcγ RI-positive cells by increasing the number of FcγRI (12). Therefore, it will be interesting to examine the correlation between the levels of each cytokine, and the severity of the diseases.

In this report, we have also described a possible role of Fcγ RII in the pathogenesis of dengue infections. We have begun to determine the classes of FcγR which can mediate ADE and the regulation of ADE by lymphokines. We recently reported that IFNγ augments antibody-dependent dengue virus infection of monocytic cells (12). We demonstrated that IFNγ increases the number of Fcγ RI detected by monoclonal antibody 32, and this increase results in the augmented uptake of dengue virus-antibody complexes, indicating that FcγRI can mediate ADE. In this report, we showed that FcγRII also mediates ADE. We used the K562 cell line, which has FcγRII but does not have FcγRI or FcγRIII, and an FcγRII-specific monoclonal antibody IV.3. Therefore, our results indicate that both FcγRI and FcγRII can mediate ADE. FcγRII is broadly expressed on monocytes, neutrophils, eosinophils, platelets and B cells, while FcγRI is only expressed on monocytes and IFNγ-treated neutrophils (4,5,8). It has been reported that FcγRII is modulated by colony stimulating factor (11), and FcγRI is modulated by IFNγ (28,29). FcγRII binds to human IgG with a subclass specificity of IgG1>IgG2= IgG4>>IgG3 while FcγRI binds with a subclass specificity of IgG1>IgG3>>IgG2 (5). These observations suggest that FcγRII may contribute to the pathogenesis of dengue virus infections in a different manner from FcγRI. There is another class of FcγR, named FcγRIII. FcγRIII is

expressed on macrophages, neutrophils, eosinophils and natural killer cells (1,2,5). The role of FcγRIII in dengue virus infection has not been described.

It is known that FcγR have various biological functions. FcγR mediates endocytosis of immune complexes (30), and antibody-dependent cell-mediated cytotoxicity (31). Binding of antigen-antibody complexes to FcγR stimulates cells to produce superoxide (32) and inflammatory mediators (33). In the present report we showed that FcγRII mediate ADE of dengue virus infection as does FcγRI. ADE has also been reported using other viruses, such as HIV (34), West Nile virus (35), yellow fever virus (36) and influenza virus (37). It is possible that ADE is a common mechanism of virus infection of FcγR-positive cells in the presence of preexisting antibodies, and this may be an important biological function of Fcγ receptors.

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