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HUMAN IMMUNE RESPONSE TO DENGUE INFECTIONS

ANNUAL REPORT

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We have mapped the epitopes recognized by three NS3-specific $CD4^+$ $CD8^-$ human cytotoxic T lymphocyte (CTL) clones. The epitope recognized by the serotype-cross-reactive clone JK34 is located within amino acids (a.a.) 251-265 of NS3. The epitopes recognized by the serotype-crossreactive clone JK32 and the flavivirus-crossreactive clone JK43 are located within a.a. 1-452 and a.a. 1-183 of NS3, respectively. We also established 16 dengue virus-specific $CD8^+$ $CD4^-$ T cell clones. The clone #2.8 recognize dengue virus types 2 and 4, and the clone #2.9 recognize four serotypes of dengue viruses. The clones #2.8 and #2.9 recognize NS3 protein, and the epitope recognized by the clone #2.8 is located within a.a. 453-618. These results indicate that NS3 contains multiple dominant T cell epitopes recognized by $CD4^+$ and $CD8^+$ human CTL.

To analyse the mechanisms of antibody-dependent enhancement, we prepared bispecific antibodies. Bispecific antibodies targeting dengue virus to β_2 microblogulin, CD15 or CD33 enhanced dengue virus infection. These results indicate that cell surface molecules other than FcR can mediate ADE and suggest that the FCγR may not provide a unique signal necessary for enhanced infection.

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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) (1-3). 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 $Fc\gamma R$-positive monocytes by antibodies to dengue viruses contributes to the pathogenesis (1-3).

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 (6). $CD4^+$ T cells proliferate and produce $IFN\gamma$, which upregulates expression of $Fc\gamma RI$ and augments dengue virus infection in the presence of antibody to dengue viruses (7). 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 $IFN\gamma$ 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 (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). We reported virus and dengue serotype-specificity and protein recognition of these clones in the previous annual report. We have also established $CD8^+$ $CD4^-$ T cell clones and lines from a donor who had been immunized with experimental live attenuated dengue-4 vaccine. In this report we describe epitopes recognized by $CD4^+$ T cell clones and our recent characterization of $CD8^+$ T cell clones.

II. RESULTS

A. Dengue virus-specific, CD4+ cytotoxic T lymphocytes (CTL)

A-1. Summary of dengue virus specific CD4+ CD8- T cell clones

Dengue virus-specific T cell clones were established from lymphocytes of donor A using limiting dilution methods as described previously (5). Seven clones were established using dengue-3 Ag, 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. Seven of 13 clones reexamined recognized NS3 protein. Table 1 shows summary of dengue virus-specific $CD4^{+}$ $CD8^{-}$ T cell clones.

Table 1: Summary of dengue virus-specific $CD4^{+}$ $CD8^{-}$ T cell clones

Virus- and serotype-specificity	Clones	HlA-restriction	Protein recognized[a] NS3	NS1
Serotype-specific				
D3	JK21	DR	not tested	
	JK37	DP	+	-
Subcomplex-specific				
D2, D3, D4	JK36	DQ	+	-
	JK46	DQ	+	-
D1, D2, D3	JK44	DR	-	-
Dengue serotype-crossreactive				
D1, D2, D3, D4	JK32	DP	+	-
	JK34	DP	+	-
	JK39	DP	+	-
	JK41	DP	-	-
Flavivirus-crossreactive				
D1, D2, D3, D4 WNV	JK28	DP	-	-
D1, D2, D3, D4 YFV, WNV	JK26	DP	-	-
	JK43	undetermined	+	-
	JK49	DP	-	-

a. + denotes recognition and - denotes no recognition

A-2. <u>Localization of epitopes on NS3 determined using dengue-vaccinia recombinant viruses</u>

We attempted to localize epitopes recognized by these clones on NS3, using dengue-vaccinia recombinant viruses. Recombinant vaccinia virus #F which contains the entire NS3 genomes, #E which codes for amino acid (a.a.) residues 1-452 of NS3, #G which codes for a.a. residues 1-182 of NS3, and #X which codes for a.a. residues 453-618 were prepared by Dr. C.-J. Lai of NIAID, NIH. JK32 lysed autologous lymphoblastoid cell line (LCL) infected with #F and #E, but did not lyse those infected with #X. JK34 lysed LCL infected with #F and #E, but did not lyse those infected with #G or #X. JK43 lysed LCL infected with #F, E# and #G, but did not lyse LCL infected with #X (Table 2). These results indicate that the epitope recognized by JK32 is located within a.a. 1-452, the epitope recognized JK34 is located within a.a. 183-452 of NS3, and the epitope recognized by JK43 is located within a.a. 1-182 of NS3.

Table 2: Localization of epitopes recognized JK34 and JK43 on NS3

Target cells infected with re-vaccinia virus (VV)	% specific ^{51}CR release[a] JK32	JK34	JK43
#D = vv (control	0	0	0
#F = vv (entire NS3 a.a. 1-618)	32	66	35
#E = vv (NS3 a.a. 1-452)	35	62	21
#G = vv (NS3 a.a. 1-182)	ND	0	18
#X = vv (NS3 a.a. 453-618)	7	4	1
Control Ag	8	0	1
Dengue-4 Ag	57	68	47

[a]Effector:target ratio was 8:1 for JK32, 15:1 for JK34 and 6:1 for JK43. 6 hours assay.

A-3. Mapping of the epitope recognized by JK34 using overlapping synthetic peptides

To further map the epitopes we synthesized 22 overlapping peptides which cover a.a. 183-452 of NS3 according to the amino acid sequencc of NS3 of dengue-4 virus, Caribbean strain 814669. JK34 lysed LCL pulsed with a peptide #4 (a.a. 251-265) (Table 3). This result indicates that the epitope recognized by JK34 is located within a.a. 251-265 of NS3.

Table 3: Recognition of a.a. 251-265 by a dengue serotype-cross reactive CD4+ T cell clones, JK34[a]

Peptide	a.a. number on NS3	% specific ^{51}Cr release
1	183-197	3.7
2	191-205	5.1
3	213-227	2.2
4	251-265	26.1
5	258-272	5.4
6	266-280	3.3
7	273-287	2.4
8	281-295	0.6
9	288-302	6.3
10	296-310	4.2
11	303-317	4.0
12	311-325	5.1
13	333-347	7.0
14	356-370	4.0
15	378-392	3.8
16	386-400	5.8
17	401-415	5.5

18	408-422	3.2
19	416-430	3.7
20	423-437	5.8
21	431-445	6.4
22	438-452	3.8
No peptide		2.5
D4V - Ag		

[a]Autologous LCL were cultured with peptides at 20 uM for 20 hours, and used as target cells. Effector:target ratio was 8:1. 6 hour assay.

B. Dengue virus-specific, HLA class I-restricted, CD8+ CTL

B-1. Lysis of dengue virus-infected fibroblasts by CTL in bulk culture

We have also analyzed dengue virus-specific $CD8^+$ $CD4^-$ CTL. PBMC from a dengue-4 virus-immune donor (#C) lysed dengue virus-infected fibroblasts after *in vitro* stimulation (Table 4). Effector cells were characterized as $CD8^+$ $CD4^-$ cells, and lysis was restricted by HLA class I. E protein and at least one of the NS2b, NS3, NS4a, or NS4b proteins were recognized by $CD8^+$ CTL in bulk culture (6).

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

PBL stimulated with	% specific 51Cr release from fibroblasts infected with: dengue-4	dengue 2	None
Dengue-4 virus	26	26	2
Dengue-2 virus	28	24	7
None	2	5	2

[a]Effector:target ratio was 40:1, 4 hour assay.

B-2. Dengue virus-specific CD8+ CTL clones

To further characterize dengue virus-specific $CD8^+$ CTL, we established 16 $CD8^+$ CTL clones and lines from PBMC of a donor #C. These CTL clones lysed dengue-2 virus-infected autologous fibroblasts, but did not lyse uninfected fibroblasts (Table 5).

Table 5: Lysis of dengue-2 virus-infected autologous fibroblasts by $CD8^+$ CTL clones[a]

Lines and clones	Effector/ Target Ratio	% specific 51CR release: Dengue-2 infected fibroblasts	Uninfected fibroblasts	K562
Long-term cultured lines				
a	3	21	1	0
b	5	10	0	0
c	10	40	0	ND
d	10	18	0	ND
e	10	51	5	ND
f	10	43	1	ND
Clones				
1.M	10	27	3	2
1.23	2	30	0	0
1.27	7	47	0	0
1.28	5	15	0	0
2.1	8	28	3	ND
2.3	9	8	0	ND
2.6	2	27	0	ND
2.7	2	26	0	ND
2.8	5	31	0	ND
2.9	1	27	0	ND

[a]6 hour assay.

Two of these CTL clones were further examined for virus and dengue serotype specificity. Clone #2.8 recognized dengue-2 and dengue-4 viruses, but did not recognize dengue-1, dengue-3, West Nile virus or yellow fever virus. Clone #2.9 recognized four serotypes of dengue viruses, but did not recognize West Nile virus or yellow fever virus (Table 6). Therefore, clone #2.8 is dengue subcomplex-specific and #2.9 is dengue serotype cross-reactive.

Table 6: Different virus- and dengue serotype-specificity of two $CD8^+$ $CD4^-$ clones[a]

Clone	% specific 51Cr release: Dengue-1	Dengue-2	Dengue-3	Dengue-4	West Nile	Yellow Fever	Control Ag
#2.8	0	<u>32</u>	0	<u>54</u>	0	0	1
#2.9	<u>77</u>	<u>88</u>	<u>96</u>	<u>100</u>	0	0	0

[a]Autologous LCL were cultured with antigens for 20 hours and used as target cells. Effector:target cells were 5:1. 6 hour assay.

B-3. HLA restriction in the lysis of target cells by dengue virus-specific CD8+ CTL clones

HLA restriction in the lysis of target cells by these CTL clones were examined, using monoclonal antibodies to HLA class I, HLA DP, HLA DQ and HLA DR. Monoclonal antibodies to HLA class I (W6/32) inhibited the lysis of target cells by all the clones examined (Table 7). Therefore, these dengue virus-specific $CD8^+$ CTL clones are HLA class I-restricted.

Table 7: HLA class I-restriction in the lysis of target cells by dengue virus-specific $CD8^+$ CTL clones[a]

Target cells	Clones	% specific 51Cr release				
		No Antibody	Anti-HLA Class I	Anti-HLA DR	Anti-HLA DQ	Anti-HLA DP
Dengue 2-infected fibroblasts						
	1.27	26	6	11	18	20
	2.8	37	17	31	31	28
	a	43	2	32	ND	34
	e	51	13	49	ND	39
Dengue 2 Ag-cultured LCL						
	2.8	71	2	70	77	71
Dengue 4 Ag-cultured LCL						
	2.8	93	0	92	89	81

[a]Effector:target ratio was 10:1, 6 hour assay. W6/32, B7/21.7, S3/4 and OKIa1 were used as anti-HLA class I, anti-HLA DP, anti-HLA DQ and anti-HLA DR, respectively.

Clone #2.8 was further examined for HLA restriction, using allogenic target cells which partially share HLA class I antigens with autologous target cells (Table 8). Clone #2.8 lysed all the dengue 4 Ag-cultured allogeneic target cells which share HLA B35. These results indicate that clone #2.8 is HLA B35 restricted.

Table 8: HLA B35-restricted lysis of target cells by a clone #2.8

Target cells	HLA class I alleles			% specific 51Cr release	
	A	B	C	Dengue 4 Ag	Control Ag
Autologous (CA)	2,23	35,44	4,4	93	0
GM3106	1,1	35,35	4,4	80	9
GM3104A	3,3	35,35	4,4	78	0
JC	3,24	35,35	4,4	78	1
Thai 1975b	2,33	44,44	3,3	0	0
GM6816	2,2	38,38	?	0	0
IK	24,24	44,44	?	0	0
66390	3,24	7,62	4,7	0	0
GM06825A	23,23	7,7	7,7	0	0

We then used Hmy2CIR cells transfected with HLA B35 or HLA B51 as target cells to confirm our observations in previous experiments. Hmy2CIR cells do not express HLA A and HLA B alleles (Table 9). These cells were cultured with dengue 4 antigen and used as target cells. Clone #2.8 lysed HLA B35-transfected Hmy2CIR cells cultured with dengue-4 Ag, but did not lyse B-51 transfected Hmy2CIR cells cultured with dengue-4 Ag. This result confirmed our conclusion that clone #2.8 lyse target cells in a HLA B35-restricted fashion.

Table 9: Lysis of dengue 4 Ag-cultured, HLA B35-transfected target cells by clone #2.8

Effector:Target ratio	% 51CR release	
	Hmy2CIR/B35	Hmy2CIR/B51
30	55	7
10	54	6
3	31	5
1	19	0

B-4. Mapping epitope recognized by dengue virus-specific CD8+ CTL clones

We are mapping epitopes recognized by dengue virus-specific $CD8^+$ CTL clones, using recombinant vaccinia viruses. Recombinant vaccinia virus #A contains dengue-4 genome which codes for NS1, NS2a, Ns2b, NS3, NS4a and NS4b proteins. Recombinant #B codes for the dengue-4 C, Pre-M, E, NS1 and NS2a. Recombinant vaccinia viruses #D, E#, #F, and #X were described above. Clone #2.8 lysed target cells infected with #A, #F and #X, and clone #2.9 lysed target cells infected with #F and #X. These results indicate that clones #2.8 and #2.9 recognize NS3, and that the epitopes which are recognized by #2.8 and #2.9 are located within a.a. 453-618 of the NS3 protein (Table 10).

Table 10: Lysis of autologous LCL infected with dengue-vaccinia recombinant viruses by $CD8^+$ CTL clones[a]

Target cells infected with	% specific 51Cr release		
	Clone #8		
	Exp. 1	Exp. 2	Clone #9
#A=VV[NS1, NS2a, NS2b, NS3, NS4a, NS4b]	29	ND	ND
#B=VV[C, pre-M, E, NS1, NS2a]	0	ND	ND
#D=vaccinia control	0	1	ND
#F=VV[whole NS3, a.a. 1-618]	28	34	14
#E=VV[NS3, a.a. 1-452]	0	0	0
EX=VV[NS3, a.a. 453-618]	ND	39	14

[a]Effector:target ratio was 10:1. 6 hour assay.

C. Antibody-dependent enhancement of dengue virus infection mediated by bispecific antibodies

Antibody-dependent enhancement of dengue virus infection has been demonstrated to occur via FcγRI (7) and FCγRII (9). Additionally, IFN-γ which up-regulates FcγRI expression (10,11), has been shown to augment ADE (7). Although some information concerning the antigenic determinants responsible for ADE of dengue virus is known (12,13), the mechanisms of ADE have not been elucidated. One possibility is that binding of virus-antibody complex to the FCγR triggers signals which are necessary to enhanced infection. Another possibility is that antibody to dengue virus directs the virus to the cell surface, thereby increasing the probability of interactions between dengue virus and its viral receptor. In ADCC, only Fcγ receptors can trigger the signals required to induce killing (14). Similarly, phagocytosis is triggered through Fc receptors and not through engagement of other cell surface molecules (15). These results indicate that specific signaling through Fc receptors is required to mediate ADCC and phagocytosis.

In this study, we examine the mechanisms of ADE by using bispecific antibodies. We prepare bispecific antibodies by chemically cross-linking anti-dengue virus antibodies to antibodies specific for cell surface molecules, including FcγRI, FcγRII, β2-microglobulin, CD15 and CD33, and examine whether these bispecific antibodies enhance dengue virus infection. Antibody dependent enhancement is mediated by bispecific antibodies targeting dengue virus to FcγRI and FcγRII. Furthermore, bispecific antibodies targeting dengue virus to the other cell surface molecules including β2-microglobulin, CD15 and CD33, also enhance dengue virus infection.

C-1. Bispecific antibodies targeting dengue virus to Fc RI enhance infection

Previously, FcγRI was shown to mediate ADE in the presence of conventional anti-dengue virus antibodies (7). Therefore, as an initial test of bispecific antibody-mediated ADE, we investigated whether targeting dengue virus to FcγRI by bispecific antibodies would enhance infection. U937 cells were incubated with IFNγ for 24 hours to up-regulate FcγR expression and infected with dengue virus-bispecific antibody complexes. The percent of dengue virus antigen-positive cells was examined by indirect immunofluorescence 24 hours after infection. The bispecific antibodies 32 x 4G2 and 32 x 2H2 both enhanced dengue virus infection (Table 11, Exp. 1-3). Viral titers from the culture supernatant fluids of U937 cells infected with dengue-bispecific antibody complex (32 x 4G2) were higher than those from the culture fluids of U937 cells infected with dengue virus alone (Table 11, Exp. 2). In the next series of experiments we used A12.13 without IFNγ-pretreatment. A12.13 cells are subclones of U937 cells which have a high expression of $Fc\gamma RI$ (16). A12.13 without $IFN\gamma$ treatment had enhanced infection with bispecific antibody complex 32 x 4G2 (Table 11, Exp. 4).

A bispecific antibody which is specific for Fc RI and Toxoplasma gondii (32 x anti-TG) did not enhance infection (Table 11, Exp. 5). Thus, enhanced infection with 32 x 4G2 and 32 x 2H2 was not due to a nonspecific effect of a bispecific antibody binding to Fc RI. Bispecific antibody 32 x 2H2 did not enhance infection of cells that do not express Fc RI including K562, Molt-3 and Raji cells (data not presented).

Table 11: Enhancement of dengue virus infection by bispecific antibodies targeting dengue virus to Fc RI[a]

	Cells	Antibody	Dilution	% Dengue Antigen Positive Cells[b]	Viral Titer (p.f.u./ml)
Exp. 1	U937	None	--	9.7	
		4G2	$1:4x10^2$	79.4*	
		32x4G2	$1:2x10^2$	47.4*	
Exp. 2	U937	None	--	18.4	$8.0x10^5$
		32x4G2	$1:2x10^2$	52.2*	$2.8x10^6$
Exp. 3	U937	None	--	5.7	
		2H2	$1:4x10^2$	76.6*	
		32x2H2	$1:2x10^3$	19.4*	
Exp. 4	A12.13	None	--	8.2	
		4G2	$1:4x10^2$	81.3*	
		32x4G2	$1:2x10^2$	24.7*	
Exp. 5	A12.13	None	--	6.8	
		2H2	$1:4x10^2$	33.5*	
		32xTg	$1:2x10^1$	4.4NS	
			$1:2x10^2$	6.0NS	
			$1:2x10^3$	5.9NS	

[a]U937 cells were pre-treated with IFN (100 u/ml) for 24 hours and then infected with dengue virus or dengue virus antibody complex. A12.13 cells were not pre-treated with $IFN\gamma$ before infection.

[b]Chi square analysis was used to compare the cells infected in the presence of anti-dengue virus antibody to those cells infected in the absence of antibody. $*p \leq 0.001$

NS not significant

The enhancement of U937 cell infection mediated by 32 x 4G2 could be significantly inhibited by preincubation of these cells with mAb 32 and $F(ab')_2$ of mAb 32 but not by mAb 251 (Table 12). These results indicate that, similar to conventional anti-dengue virus antibodies, bispecific antibodies targeting dengue virus to FcγRI can enhance infection.

Table 12: Monoclonal antibody to FcγRI inhibits ADE mediated by bispecific antibody targeting dengue virus to FcγRI[a]

	Cells	Antibody	Blocking Antibody	% Dengue Antigen positive cells[b]
Exp. 2	U937	None	None	6.0
		32x4G2	None	28.8
			32	25.5*
			$F(ab')_2$ of 32	16.7*
			251	29.9NS

[a]U937 cells were pre-treated with IFNγ (100 u/ml) for 24 hours, washed and then incubated with mouse IgG1 mAb 32 (100 ug/ml), $F(ab')_2$ of mAb 32 (100 ug/ml), or mouse IgG1 mAb 251 (100 ug/ml) for 1 hour and infected with dengue virus in the presence of bispecific antibody 32 x 4G2 at a concentration of 1:200.

[b]The cells infected with dengue virus-bispecific antibody complex 32 x 4G2 that were incubated with a blocking antibody were compared using Chi square analysis to the cells not incubated with a blocking antibody. *$p \leq 0.001$, NS not significant

C-2. <u>Bispecific antibodies targeting dengue virus to FcγRII enhance infection</u>

Conventional antibodies to dengue virus can also mediate ADE through FcγRII (9). U937 and K562 cells infected with dengue virus complexed to a bispecific antibody directed against Fc RII (IV.3 x 2H2) demonstrated enhancement of dengue virus infection (Table 13, Exp. 1-3). IFNγ which does not modulate the expression of FcγRII on these cells, had no significant effect on the infection of K562 cells with dengue virus-specific antibody complex (IV.3 x 2H2) (Table 13, Exp. 2). Viral titers from the culture supernatant fluids of K562 cells infected with dengue virus-bispecific antibody complex (IV.3 x 2H2) were higher than those from the culture fluids of cells infected with dengue virus alone (Table 13, Exp. 2).

A bispecific antibody linking FcγRII to <u>Toxoplasma gondii</u> (IV.3 x anti-Tg) did not mediate enhancement of dengue virus infection in K562 cells (Table 13, Exp. 3). Furthermore, the bispecific antibody IV.3 x 2H2 did not enhance infection of Molt-3 cells, which do not express FcγRII (data not presented).

Table 13: Enhancement of dengue virus infection by bispecific antibodies targeting dengue virus to Fc RII

	Cells	Antibody	Dilution	% Dengue Antigen Positive Cells[b]	Viral Titer (p.f.u./ml)
Exp. 1	U937[a]	None	--	5.7	
		2H2	$1:4x10^2$	76.6*	
		IV.3x2H2	$1:2x10^1$	9.1*	
			$1:2x10^2$	37.9*	
			$1:2x10^3$	6.2 NS	
Exp. 2	K562	None	--	4.9	$9.0x10^4$
		IV.3x2H2	$1:2x10^2$	15.1*	$3.0x10^5$
	K562[a]	None	--	3.3	$1.6x10^4$
		IV.3x2H2	$1:2x10^2$	18.1*	$3.0x10^5$
Exp. 3	K562	None	--	11.8	
		IV.3x2H2	$1:2x10^2$	50.4*	
		IV.3xTg	$1:2x10^1$	9.0 NS	
			$1:2x10^2$	11.4 NS	
			$1:2x10^3$	11.8 NS	

[a]U937 or K562 cells were pre-treated with IFNγ (100 u/ml) for 24 hours and then infected with dengue virus or dengue virus antibody complex.
[b]Chi square analysis was used to compare the cells infected in the presence of anti-dengue virus antibody to those cells infected in the absence of antibody. *$p \leq 0.001$, NS not significant

The enhancement of K562 cell infection mediated by the bispecific antibody IV.3 x 2H2 was significantly inhibited by preincubation of these cells with mAb IV.3, but not by mAb 32 nor mAb BBM1 (Table 14). These results indicate that bispecific antibodies targeting dengue virus to Fc RII also enhance dengue virus infection.

Table 14: Monoclonal antibody to FcγRII inhibits ADE mediated by bispecific antibody targeting dengue virus to Fc RII[a]

Cells	Antibody	Blocking Antibody	% Dengue Antigen Positive Cells[b]
K562	None	None	27.6
	IV.3x2H2	None	88.0
		IV.3	34.3*
		32	86.2NS
		BBM1	84.6NS

[a]K562 cells were incubated with mouse IgG2b mAb IV.3 (100 ug/ml), mouse IgG1 mAb 32 (100 ug/ml) or mouse IgG2b mAb BBM1 (100 ug/ml) for 1 hour, and then infected with dengue virus at an m.o.i. of 70

p.f.u./cell in the presence of bispecific antibody IV.3 x 2H2 (1:2 x 10^2).

[b]The cells infected with dengue virus-bispecific antibody complex IV.3 x 2H2 that were incubated with a blocking antibody were compared using Chi square analysis to the cells not incubated with a blocking antibody. *$p \leq 0.001$, NS not significant

C-3. <u>Bispecific antibodies targeting dengue virus to cell surface molecules other than Fcγ receptors enhance infection</u>

The above experiments demonstrated that bispecific antibodies which link dengue virus to FcγRI or FcγRII mimic conventional antibodies in enhancing infection. The advantage of using bispecific antibodies is that they permit targeting to any antigen on the cell surface. Therefore, in order to investigate if the FcγR has a unique function in ADE, infections were performed in the presence of bispecific antibodies targeting dengue virus to non-FcγR molecules.

We examined ADE mediated by a bispecific antibody targeting dengue virus to the β_2-microglobulin of class I major histocompatibility complex (MHC) molecule on U937 cells and the FcγR negative T cell line Molt-3. IFNγ has been shown to up-regulate MHC class I (17) and therefore, both U937 and Molt-3 cells were incubated with IFNγ before infection. In these cells, targeting dengue virus to β_2-microglobulin with the bispecific antibody BBM1 x 2H2 enhanced infection (Table 15, Exp. 1-5). Viral titers from the culture fluids of Molt-3 cells infected with dengue virus-bispecific antibody complex (BBM1 x 2H2) were higher than those in the culture fluids of cells infected with dengue virus alone (Table 16, Exp. 4). Consistent with the observation that Molt-3 cells do not express Fc receptors, the parent IgG2a mAb, 2H2, did not mediate ADE of these cells (Table 15, Exp. 2).

The control bispecific antibody, BBM1 x anti-Tg, did not mediate enhanced dengue virus infection of Molt-3 cells (Table 15, Exp. 5). Daudi cells, which do not express β_2-microglobulin, did not show enhanced infection in the presence of BBM1 x 2H2 (data not presented). Furthermore, the enhancement of Molt-3 infection mediated by bispecific antibody BBM1 x 2H2 was significantly inhibited by preincubation of these cells with the mAb BBM1 (Table 17, Exp. 1).

Table 15: Enhancement of dengue virus infection by bispecific antibodies targeting dengue virus to ß2-microglobulin

	Cells	Antibody	Dilution	% Dengue Antigen Positive Cells[b]	Viral Titer (p.f.u./ml)
Exp. 1	U937[a]	None	--	5.7	
		2H2	$1:4x10^2$	76.6*	
		BBM1x2H2	$1:2x10^1$	13.7*	
			$1:2x10^2$	22.6*	
			$1:2x10^3$	6.3 NS	
Exp. 2	Molt3[a]	None	--	7.4	
		2H2	$1:4x10^2$	2.2*	
		BBM1x2H2	$1:2x10^1$	5.5 NS	
			$1:2x10^2$	20.0*	
			$1:2x10^3$	10.7 NS	
Exp. 3	Molt3	None	--	3.7	
		BBM1x2H2	$1:2x10^2$	9.7**	
	Molt3[a]	None		3.8	
		BBM1x2H2	$1:2x10^2$	20.2*	
Exp. 4	Molt3[a]	None	--	0.8	$2.0x10^2$
		BBM1x2H2	$1:2x10^2$	7.8*	$1.1x10^3$
Exp. 5	Molt3[a]	None	--	5.0	
		BBM1x2H2	$1:2x10^2$	11.2*	
		BBM1xTg	$1:2x10^1$	5.0 NS	
			$1:2x10^2$	4.3 NS	
			$1:2x10^3$	6.2 NS	

[a]U937 or Molt-3 cells were pre-treated with IFN (100 u/ml) for 48 hours and then infected with dengue virus or dengue virus antibody complex. The m.o.i. used in Exp. 4 was 14 p.f.u./cell.

[b]Chi square analysis was used to compare the cells infected in the presence of anti-dengue virus antibody to those cells infected in the absence of antibody. $*p \leq 0.001$, $**p \leq 0.05$, NS not significant.

We then examined bispecific antibodies targeting dengue virus to two other molecules, CD15 and CD33, for their ability to enhance infection. HL60 cells infected in the presence of a bispecific antibody which targeted the virus to CD15 (PM81 x 4G2) also displayed enhanced infection demonstrated by an increase in the percentage of dengue Ag-positive cells and by the viral titers (Table 16, Exp. 1 and 2). This enhancement was blocked by preincubation with mAb PM81 but not by mAb Leu 11b (Table 17, Exp. 2).

The bispecific antibody 251 x 4G2 which targets dengue virus to CD33 also showed enhancement of infection of U937 cells demonstrated by the percentage of dengue Ag-positive cells and by the viral titers (Table 16, Exp. 3 and 4). This enhancement was blocked by preincubation of U937 cells with mAb 251, but not mAb 32 (Table 17, Exp. 3). Taken together, these results indicate that bispecific antibody-mediated ADE can occur through cell surface molecules other than FcγR.

Table 16: Enhancement of dengue virus infection by bispecific antibodies targeting dengue virus to CD15, and targeting dengue virus to CD33

	Cells	Antibody	Dilution	% Dengue Antigen Positive Cells[a]	Vital Titer (p.f.u./ml)
Exp. 1	HL60	None	--	0.6	
		PM81x4G2	$1:2x10^1$	4.6*	
			$1:2x10^2$	6.6*	
			$1:2x10^3$	14.7*	
Exp. 2	HL60	None	--	0.4	$1.0x10^1$
		PM81x4G2	$1:2x10^4$	13.5*	$2.6x10^3$
Exp. 3	U937	None	--	5.7	
		251x4G2	$1:2x10^1$	4.9 NS	
			$1:2x10^2$	19.2*	
			$1:2x10^3$	16.1*	
Exp. 4	U937	None	--	4.0	$1.1x10^3$
		251x4G2	$1:2x10^2$	22.5*	$1.8x10^4$

[a]Chi square analysis was used to compare the cells infected in the presence of anti-dengue virus antibody to those cells infected in the absence of antibody. *$p \leq 0.001$, NS not significant.

NS not significant

Table 17: Inhibition of bispecific antibody-mediated ADE by monoclonal antibodies to ß2-microglobulin, CD15 and CD33[a]

	Cells	Antibody	Blocking Antibody	% Dengue Antigen Positive Cells[b]
Exp. 1	Molt3	None	None	10.5
		BBM1x2H2	None	20.7
			BBM1(300 ug/ml)	6.0*
			BBM1 (30 ug/ml)	7.5*
			NMS (50 ug/ml)	19.9 NS

Exp. 2	HL60	None	None	0.7
		PM81x4G2	None	12.8
			PM81 (3.5 ug/ml)	2.2*
			(0.35 ug/ml)	3.6*
			Leu11b (2.5 ug/ml)	12.9 NS
			Leu11b (0.25 ug/ml)	11.0 NS
Exp. 3	U937	None	None	4.0
		251x4G2	None	22.5
			251 (100 ug/ml)	9.3*
			32 (100 ug/ml)	25.6 NS

[a]Molt-3 cells were pre-treated with IFNγ(100 u/ml) for 48 hours, washed and then incubated with mouse Ig2b mAb BBM1 or normal mouse serum (NMS) for 1 hour and then infected with dengue virus in the presence of bispecific antibody BBM1 x 2H2 (1:2 x 10^2). HL60 cells were incubated with mouse IgM mAb PM81 or mouse IgM mAb Leu11b and U937 cells were incubated with mouse IgG1 mAb 251 or mouse IgG1 mAb 32 for 1 hour, and then infected with dengue virus in the presence of bispecific antibody PM81 x 4G2 (1:2 x 10^4) or 251 x 4G2 (1:2 x 10^2) respectively.

[b]The cells infected with dengue virus-bispecific antibody complex that were pre-incubated with a blocking antibody were compared using Chi square analysis to the cells not incubated with a blocking antibody. *$p \leq 0.001$, NS not significant.

III. DISCUSSION

In this paper, we describe the establishment of dengue virus-specific, serotype cross-reactive, human CD8+ CD4- CTL lines and clones. We utilized these CTL to characterize serotype cross-reactive cytotoxicity, allele specificity, and the epitopes recognized during class I restricted human T cell response to dengue virus infection. All human CTL lines and clones were established from the PBMC of a dengue 4 virus-immune donor obtained two years post-infection. Live dengue 4 virus at high titers in the presence of autologous PBMC was necessary for establishment and maintenance of class I restricted CTL. Several clones and lines were maintained for greater than 1 year in continuous culture using this method. However, the majority of clones grew slowly and were relatively short-lived (<2 months in continuous culture) leaving only a limited number of lines/clones available for advanced characterization. Class I restricted activity was initially screened for using ^{51}Cr-release cytotoxicity assays with autologous, dengue 2 virus infected fibroblasts as targets. Fibroblasts lack constitutive HLA class II expression in the absence of IFNγ, and no class II expression was detected by FA analysis using FITC-conjugated anti-class II antibody. The class I restricted nature of 2 lines and 2 clones was further demonstrated by inhibition of lysis of autologous D2Vinfected target cells by an anti-class I specific antibody (W6/32), but not by anti-class II specific antibodies directed against DR, DQ, or DP antigens.

Our results indicate that NS3 provides immunodominant epitope(s) for the dengue-virus specific, serotype cross-reactive, class I restricted CTL response in this donor and that multiple epitopes are contained in amino acids 493-618 of NS3. Dengue virus immune responses in more donors need to be characterized to determine the relative contribution of NS3 in generating CTL response to dengue virus infection. The importance of NS3 is supported by other recent work from our laboratory. CTL clones recognizing NS3 epitopes also dominate the dengue virus specific, serotype cross-reactive, class II restricted T cell memory of a dengue 3 virus-immune donor. It is not known why NS3 appears to provide dominant epitopes for memory CTL response to dengue virus infection. Our results suggest that NS3 may contain dominant T cell epitopes for both $CD8^+$ and $CD4^+$ T cell responses. Therefore, NS3 or peptides of NS3 may provide plausible candidates for design of effective dengue virus subunit vaccines.

Antibody responses to viruses are important for controlling infections, however, dengue virus antibodies at subneutralizing concentrations enhance infection. This phenomenon called antibody-dependent enhancement (ADE) has been shown to be mediated by FcγRI (7) and FcγRII (9). Epidemiologic studies have suggested that ADE may be responsible for the severe manifestations of dengue virus infection including dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) (1-3). However, neither the mechanism of ADE nor the role of FcγR in ADE has been clearly elucidated. Electron microscopic (EM) studies of another flavivirus, West Nile Virus, have shown that virus or virus-antibody complex enters cells via coated pits (18). Dengue virus that is not coated with antibody has been demonstrated by EM studies to directly fuse with the cell membrane (19). However, the precise mechanism of dengue virus entry into cells in the presence of antibody is unknown.

Bispecific antibodies have proved useful in defining the functions of the different Fcγ receptors in mediating cytotoxicity and phagocytosis (14,15). ADCC by human monocytes, granulocytes and NK cells requires specific interaction of antibody with an Fcγ R for lysis to occur (14). Effector-target conjugates formed by bispecific antibodies which do not engage an Fc R do not lead to killing (14). Similarly, human alveolar macrophage ADCC was mediated via bispecific antibodies which target to FcγRI, FcγRII or FcγRIII but not via a bispecific antibody which targets to other molecules such as ß2-microglobulin (20). Using this same approach, phagocytosis of bispecific antibody coated ox erythrocytes was mediated by mononuclear cells via FcγRI, FcγRII or FcγRIII but not via ß2-microglobulin (15). These findings suggest that ADCC and phagocytosis involve specific signaling provided by $Fc\gamma$ receptors.

In this study, bispecific antibodies were designed to target dengue virus to specific antigens, both $Fc\gamma R$ and non-$Fc\gamma$ receptor cell surface molecules. All of these bispecific antibodies mediated ADE of dengue virus infection in the appropriate monocytic, B cell and T cell lines. The specificity of the enhancement with bispecific antibodies was demonstrated by employing antibody blocking experiments, cell lines lacking specific cell surface antigens, and bispecific antibodies directed against an irrelevant organism.

A variety of non-$Fc\gamma R$ cell surface molecules (ß2-microglobulin, CD15, CD33) could function in ADE as well as $Fc\gamma R$, therefore, these results suggest that Fc receptors do not have a unique signaling function in ADE. These experiments, however, did not evaluate cytoplasmic signaling through each of these molecules, so a signaling requirement cannot be ruled out. Further studies utilizing cells transfected with $Fc\gamma R$ genes which lack cytoplasmic domains and do not signal will address this question. We propose that directing dengue virus to the cell surface with a bispecific antibody to an $Fc\gamma R$ or non-$Fc\gamma R$ surface molecule focuses the virus on the cell membrane and permits it to bind to its receptor more efficiently, thus increasing its infectivity. in vivo, antibody and $Fc\gamma R$ may function in enhancement of dengue virus infection by merely capturing the virus and focusing it at the cell surface.

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