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DENGUE VIRUS-SPECIFIC HUMAN T CELL CLONES

Serotype Crossreactive Proliferation, Interferon γ Production, and Cytotoxic Activity

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The pathogenesis of the severe complications of dengue virus infections, dengue hemorrhagic fever (DHF)[1] and dengue shock syndrome (DSS), is one of the most important subjects to be elucidated in dengue virus research. Epidemiological studies have shown that DHF/DSS is much more commonly observed during secondary dengue infections than primary infections (1, 2), and secondary infections are caused by virus strains of a different serotype than the dengue virus that caused the primary infection (1). It has been reported that antidengue antibodies at subneutralizing concentrations augment dengue virus infection of Fcγ receptor–positive cells, such as monocytes (3, 4). Dengue antigen–positive monocytic cells are found in patients with DHF/DSS (5, 6) and dengue virus has been recovered from the monocytic fraction of PBMC obtained from infected patients (7). Based on these observations it has been hypothesized that dengue serotype crossreactive antibodies may increase the number of dengue virus–infected monocytes during secondary infections, and lysis of these dengue-infected monocytes by immune cytolysis may lead to the pathogenesis of DHF/DSS (1, 8). Despite this hypohesis, dengue virus–specific T lymphocyte responses in humans have not been defined. We recently reported the presence of dengue antigen–specific CD4$^+$ T cells in bulk cultures of lymphocytes from dengue antibody–positive donors, which proliferate and produce IFN-γ after stimulation with dengue antigens (9). In addition, we reported that the IFN-γ produced was able to augment dengue virus infection of monocytic cells in the presence of anti-dengue antibody (10). To further elucidate the role of T lymphocytes in dengue virus infections, we have begun to analyze the serotype specificity, IFN-γ production, and cytotoxic activity of dengue antigen–specific T lymphocytes at the clonal level, using PBL of a donor who was known to have been infected with dengue 3 virus. All the clones established have a CD3$^+$ CD4$^+$ CD8$^-$ phenotype, and most of the clones are serotype crossreactive. All but one of these serotype crossreactive clones produce

[1] *Abbreviations used in this paper:* DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; LCL, lymphoblastoid cell lines; m.o.i., multiplicity of infection.

IFN-γ after stimulation with dengue virus antigens of heterologous serotypes, and the same clones lyse dengue 2 virus–infected autologous lymphoblastoid cells. These results suggest that dengue serotype crossreactive CD4$^+$ T lymphocytes may contribute to the pathogenesis of DHF/DSS by producing IFN-γ and lysing dengue virus–infected autologous cells during secondary infections.

Materials and Methods

Human PBMC. Peripheral blood specimens were obtained from a donor who had been immunized with yellow fever vaccine 2 yr earlier and was infected with dengue 3 virus, strain CH53489, 1 yr previously (11). PBMC were also obtained from a donor in Aruba who had been infected with dengue 1 virus 4 mo previously, and from a healthy blood bank donor from Massachusetts who did not have detectable antibodies to dengue virus as determined by a plaque reduction neutralization test (12). PBMC were separated by a Ficoll-Hypaque density gradient centrifugation method (13). Cells were resuspended at the concentration of 10^7/ml in RPMI containing 10% FCS (Gibco Laboratories, Grand Island, NY) and 10% DMSO (Fisher Scientific Co., Pittsburgh, PA), and were cryopreserved until use (12).

Viruses. The dengue virus strains used were type 1, the Hawaii strain; type 2, the New Guinea C strain; type 3, the CH53489 strain; and type 4, the 814669 strain. Dengue virus types 1 and 2 were supplied by Dr. Walter E. Brandt of Walter Reed Army Institute of Research, Washington DC, type 3 was supplied by Dr. Bruce L. Innis of the Armed Forces Research Institute of Medical Science, Bangkok, Thailand, and type 4 was supplied by Jack McCown of the Walter Reed Army Institute of Research.

Preparation of Dengue Antigens. Dengue antigens were prepared using dengue virus–infected Vero cells as previously reported (9). Vero cells were infected with dengue virus at an approximate multiplicity of infection (m.o.i.) of 1 plaque-forming unit (PFU)/cell, and cultured in MEM containing 2% FCS. When 50% of the monolayer developed cytopathic effects, the cells were removed using cell scrapers (Costar, Cambridge, MA), washed three times with PBS at 4°C, treated with 0.025% glutaraldehyde (Sigma Chemical Co., St. Louis, MO) in PBS for 15 min at 4°C, washed again three times with PBS, and resuspended in RPMI. They were then sonicated with a sonic dismembrator (Fisher Scientific Co.) and centrifuged at 2,500 rpm for 10 min. The supernatant fluids were collected and used as dengue antigens. Control antigens were prepared in a similar manner using uninfected Vero cells. 3 ml of antigen was obtained from 15 75-cm^2 flasks (Costar) of confluent Vero cells.

Induction of Proliferative Responses of PBMC. Proliferative responses of PBMC were detected as previously reported (9). 2 × 10^5 PBMC were cultured with dengue antigens diluted 1:30 in 0.2 ml RPMI containing 10% human AB serum (Hazleton Research Products, Inc., Lenexa, KS) and 5 × 10^{-5} M 2-ME (Sigma Chemical Co.) in 96-well round-bottomed plates (Costar) at 37°C for 6 d. Cells were pulsed with 1.25 μCi of [^{3}H]TdR for 8 h before harvest. Cells were harvested using a Titertek Multiharvester (Skatron, Inc., Sterling, VA) and [^{3}H]TdR incorporation was counted in a liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL).

Establishment of Antigen-specific T Cell Clones Using a Limited Dilution Method. 4 × 10^5 PBMC were cultured with dengue 3 antigen at a final dilution of 1:30 in 0.2 ml RPMI containing 10% human AB serum in 96-well round-bottomed plates for 7 d. On day 7, blast cells were enriched by Ficoll-Hypaque density gradient centrifugation and were cultured at concentrations of 30, 10, 3, and 1 cell/well with γ-irradiated (3,000 rad) autologous PBMC (10^5) in 0.2 ml RPMI containing 10% human AB serum, 10% IL-2 (Cellular Products, Inc., Buffalo, NY), and dengue 3 antigen at a final dilution of 1:30 in 96-well round bottomed plates. On day 14, 0.1 ml of medium was removed from each well and 10^5 γ-irradiated autologous PBMC in 0.1 ml of fresh medium with human AB serum, IL-2, and dengue antigen was added to maintain the same final concentrations described above. On day 21, cells in wells demonstrating growth were transferred to 48-well flat-bottomed plates (Costar) and were further cultured with 10^6 γ-irradiated autologous PBMC in 1 ml of RPMI containing 10% human AB serum, 10% IL-2, and dengue antigen at a dilution of 1:30.

Proliferative Responses of T Cell Clones. 10^4 T cells were cultured with 2 × 10^5 γ-irradiated

(3,000 rad) autologous PBMC in 0.2 ml RPMI containing 10% human AB serum and dengue 3 antigen diluted at 1:30 in 96-well round-bottomed plates at 37°C for 3 d. Cells were pulsed with 1.25 μCi [^{3}H]TdR for 8 h before harvest. They were harvested using a multiharvester and [^{3}H]TdR incorporation was counted in a liquid scintillation counter.

Phenotypic Analysis.   Anti-Leu-2 (CD8) antibody reacts with suppressor/cytotoxic T cells (14). Anti-Leu-3 (CD4) antibody reacts with helper/inducer T cells (14). Anti-Leu-4 (CD3) antibody reacts with pan T cells (15). Anti-Leu-2, -Leu-3, and -Leu-4 antibodies were purchased from Becton Dickinson & Co. (Mountain View, CA). Clones were stained with mAbs conjugated with FITC by direct immunofluorescence methods as described earlier (16). The percentage of antigen-positive cells was determined using a FACS (440; Becton Dickinson & Co.).

Immunoassays for IFN-γ.   Sandwich-type ELISAs were used for the estimation of IFN-γ activity as previously reported (9). Purified rabbit polyclonal anti-recombinant human IFN-γ (17) was coated on U-bottomed wells of polyvinyl chloride microtiter plates (Dynatech R/D Co., Cambridge, MA). After washing, serial dilutions of human IFN-γ standard (British standard 82/587; 3,000 IU/ampule) or culture fluid samples were added. Then, purified anti-human IFN-γ mAb 4SB3 (18) was added. Color was developed using biotinylated sheep anti-mouse Ig (Amersham International, Amersham, UK) and streptavidin biotinylated horseradish peroxidase complex (Amersham International). Levels of IFN-γ in culture fluid samples were interpolated from the IFN-γ standard calibration curve. The detection limit of the IFN-γ-specific ELISA was 0.5 IU/ml.

Preparation of Cell Lines Pulsed with Dengue Antigens.   Lymphoblastoid cell lines (LCL) were established by infecting PBMC with EBV from an infected marmoset cell line supernatant as described (19). All the transformed cells were cultured in RPMI containing 10% FCS. EBV was provided by Dr. Takeshi Sairenji of University of Massachusetts Medical Center.

4 $\times$ 10^5 LCL were incubated with dengue and yellow fever antigens at final dilution of 1:100 in 1 ml RPMI containing 10% FCS for 16 h. Cells were washed twice with RPMI/10% FCS, ^{51}Cr-labeled, and used as target cells.

Preparation of Dengue 2 Virus-infected LCL.   5 $\times$ 10^5 LCL were infected with dengue 2 virus at an m.o.i. of 5 PFU/ml for 2 h at 37°C, and resuspended in 5 ml of RPMI/10% FCS. These cells were maintained for 3-4 wk and examined for cytoplasmic dengue antigens using FA staining (12). When the percentage of dengue antigen-positive cells was >50%, they were used as target cells.

Cytotoxicity Assays.   Target cells (0.5-2 $\times$ 10^6) were labeled with 0.5 mCi of ^{51}Cr (Na$_2$CrO$_4$) (New England Nuclear, Boston, MA) in 0.2 ml of RPMI containing 10% FCS at 37°C for 60 min. Labeled cells were washed three times and suspended at 2.5 $\times$ 10^4/ml in RPMI/10% FCS. 2.5 $\times$ 10^3 cells in 0.1 ml were added to each well in round-bottomed microtiter plates (Linbro Chemical Co., Hamden, CT). Various concentrations of effector cells in 0.1 ml of RPMI/10% FCS were added to each well to give the described E/T ratios. After incubation at 37°C for 4 h, the supernatant fluid was collected from each well and counted in an automatic gamma counter. The percent specific ^{51}Cr release was calculated by the formula: 100 $\times$ (cpm experimental release − cpm spontaneous release)/(cpm maximal release − cpm spontaneous release).

Antibody Blocking of the Lysis of Dengue 2-infected Target Cells.   mAbs OKIa1, B7/21.7, and S3/4 recognize HLA DR, DP, and DQ determinants, respectively. mAb W6/32 recognizes a framework determinant of HLA-A, -B, and -C. B7/21.7 and S3/4 were kindly provided by Dr. Nancy Reinsmoen of University of Minnesota, Minneapolis, MN. OKIa1 and W6/32 were provided by Dr. John Sullivan of University of Massachusetts Medical Center. 2.5 $\times$ 10^3 ^{51}Cr-labeled target cells in 0.1 ml were incubated with 0.05 ml of 1:20 diluted mAbs for 30 min. The effector cells were then added in 0.05 ml and incubated for 4 h. The percentage of specific ^{51}Cr release was determined as described above.

Results

Proliferative Responses of Donor A PBMC to Dengue Antigens in a Bulk Culture.   The PBMC from donor A, who had been infected 1 yr earlier with dengue virus type

3, were cultured with dengue antigens of four serotypes, and [³H]TdR incorporation was examined. PBMC from this donor responded to dengue 3 antigen, and they also responded to dengue 1, 2, and 4 antigens to lower but significant levels. PBMC from donor B, who had been infected with dengue 1 virus ∿4 mo earlier, responded best to dengue 1 antigen and also responded to dengue antigens of other serotypes to lower levels (Table I). These results indicate that T cell proliferation in bulk culture after infection with one serotype of dengue virus is primarily serotype specific, but also contains serotype crossreactive memory responses.

Establishment of CD4⁺ T Cell Clones that Respond to Dengue 3 Antigen.   We tried to establish dengue-specific T cell clones by a limiting dilution method using lymphocytes from donor A and dengue 3 antigen. 12 clones were established that respond to dengue 3 antigen, but not to control antigen. All the clones were established from wells containing 1 cell/well. The cloning efficiency was 15% with 1 cell/well, 48% with 3 cells/well, 90% with 10 cells/well, and 100% with 30 cells/well. The clonality of the clones used in the experiments was >96%.

Phenotypic analysis of the clones using mAbs showed that all the clones have CD3⁺, CD4⁺ and CD8⁻ phenotypes (Table II).

Serotype Specificity of the Dengue-specific T Cell Clones.   These clones were examined for serotype specificity using dengue antigens of four serotypes and yellow fever antigen. A dose-response study using clone JK31 indicated that each of the antigens induced maximum proliferative responses at a 1:30 dilution (data not presented). Eight clones responded to dengue 1, 2, and 4 antigens to about the same level as to dengue 3 antigen (Table III), and therefore, they are dengue serotype crossreactive. Three of these serotype crossreactive clones (JK27, JK32, and JK35) also responded to yellow fever virus antigens. Four other clones responded predominantly to dengue 3 antigens, although there are some minor responses to other serotypes; therefore, they are serotype specific.

Production of IFN-γ by Dengue-specific T Cell Clones after Stimulation with Dengue An-

TABLE I

Proliferation Responses of the PBMC of Donor A to Dengue Antigens in Bulk Cultures

Antigens	[³H]TdR Incorporation		
	Donor A*	Donor B‡	Donor C§
		cpm	
Dengue 1	5,128	15,932	481
Dengue 2	6,643	5,983	516
Dengue 3	25,177	6,772	682
Dengue 4	2,883	4,329	603
Control	660	1,065	460
No antigen	707	1,091	450

2 × 10⁵ PBMC were cultured with dengue and control antigens diluted at 1:30 for 6 d. Cells were pulsed with 1.25 μCi [³H]TdR for 8 h and [³H]TdR incorporation was counted.
* Donor A was known to have been infected with dengue 3 virus.
‡ Donor B was known to have been infected with dengue 1 virus.
§ Donor C is from Massachusetts and does not possess any antidengue antibodies.

TABLE II

Phenotypic Analysis of Dengue Virus Antigen-specific T Cell Clones

Clones	Percent antigen-positive cells		
	CD3	CD4	CD8
JK24	97	96	1
JK26	94	96	2
JK27	97	95	0.4
JK28	93	96	0.4
JK30	98	85	0.8
JK31	99	99	0.2
JK32	99	96	3
JK33	97	95	0.2
JK34	98	92	4
JK35	99	98	4
JK36	80	94	2
JK37	98	96	1

Clones were stained with anti-CD3 (anti-Leu-4), anti-CD4 (anti-Leu-3), and anti-CD8 (anti-Leu-2) mAbs. The percentage of positive cells was counted on a FACS.

tigens. We have reported that IFN-γ augments dengue virus infection of human monocytic cells in the presence of antidengue virus antibody (10), and have hypothesized that IFN-γ may contribute to the pathogenesis of DHF/DSS (9, 10).

The dengue-specific clones were examined for INF-γ production after stimulation with dengue and yellow fever antigens (Table IV). All the serotype crossreactive

TABLE III

Proliferative Responses of Dengue Virus Antigen-specific T Cell Clones
to Dengue and Yellow Fever Antigens

Serotype	Clones	[³H]TdR Incorporation						
		Dengue 1	Dengue 2	Dengue 3	Dengue 4	Yellow fever	Control antigen	No antigen
		cpm						
Crossreactive	JK26	1,438	2,078	2,375	1,394	641	436	708
	JK27	6,683	5,711	11,728	3,839	1,880	827	650
	JK28	1,949	2,286	2,600	1,681	788	684	633
	JK32	1,416	3,290	4,102	2,410	1,925	133	245
	JK33	4,530	8,152	12,756	6,286	3,070	1,161	1,949
	JK34	2,651	8,457	9,256	4,253	1,910	480	1,076
	JK35	3,507	4,372	6,260	5,025	3,854	2,403	1,290
	JK36	1,732	13,159	15,055	7,167	903	140	1,225
Specific	JK24	2,581	2,205	13,809	1,457	355	348	108
	JK30	821	896	3,246	721	276	112	218
	JK31	745	1,269	7,309	837	397	140	133
	JK37	4,478	6,958	26,892	5,827	2,546	1,223	1,985

10^4 cells were cultured with 2×10^5 γ-irradiated autologous PBMC in 0.2 ml RPMI/10% human AB serum containing dengue, yellow fever, and control antigens diluted at 1:30 for 72 h. Cells were pulsed with 1.25 μCi [³H]TdR for 8 h before harvest.

Table IV

*IFN-γ Production by Serotype Crossreactive T Cell Clones
after Stimulation with Dengue Antigens*

Clones	IFN-γ						
	Dengue 1	Dengue 2	Dengue 3	Dengue 4	Yellow fever	Control antigen	No antigen
	U/ml						
JK26	26	74	39	19	9	6	7
JK27	7	6	35	4	3	3	3
JK28	13	14	13	6	2	1	<1
JK32	12	23	18	11	6	3	3
JK33	14	24	41	17	8	2	5
JK34	6	22	23	10	4	2	3
JK35	17	33	51	21	10	<1	3
JK36	2	15	18	7	2	3	<1

10^4 cells were cultured with 2×10^5 γ-irradiated autologous PBMC in 0.2 ml RPMI/10% human AB serum containing dengue, yellow fever, and control antigens diluted at 1:30 for 72 h. Culture fluids were collected and assayed for IFN-γ by ELISA.

clones produced IFN-γ after stimulation with dengue 3 antigen, and all but JK27 produced IFN-γ to the same or lower levels after stimulation with dengue antigens of the other serotypes. Clone JK35, which responded to yellow fever antigen, produced IFN-γ after stimulation with yellow fever antigen. Serotype-specific clones produced IFN-γ after stimulation with dengue 3 antigen (data not presented). These results suggest that IFN-γ is produced by serotype crossreactive T cells during secondary infections with different serotypes of dengue virus from primary infections.

Cytotoxic Activities of Dengue-specific T Cell Clones to Autologous LCL Pulsed with Dengue Antigens. Two serotype crossreactive 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 V). JK36, which did not proliferate after

Table V

*Cytotoxic Activities of Dengue Virus-specific T Cell Clones
to Autologous LCL Pulsed with Dengue Antigens*

Autologous LCL pulsed with antigen	Percent specific ^{51}Cr 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

2.5×10^3 target cells were incubated with effector cells for 4 h. Percent specific ^{51}Cr release was calculated by the formula described in Materials and Methods. E/T ratio was 6:1 for JK34, 5:1 for JK36, and 2:1 for JK37.

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 crossreactivity in cytotoxicity is consistent with the responses observed in proliferation assays.

Time Course and Dose-Response Studies of the Lysis of Dengue 2-infected Cells by a T Cell Clone. We then used dengue 2 virus-infected autologous LCL as target cells in cytotoxic assays. A time course study using JK32 as effector cells showed that low levels of lysis were observed as early as 2 h, and the percentage of lysis reached almost maximum levels by 4 h (Fig. 1). Significant lysis of target cells by clone JK32 was observed even at an E/T ratio of 0.3 (Table VI). JK32 did not lyse uninfected autologous LCL or K562 cells, which are sensitive target cells for human NK cells.

Lysis of Dengue 2 Virus-infected Autologous Lymphoblastoid Cell Line by Dengue Serotype Crossreactive T Cell Clones. We examined other clones for their cytotoxic activities to dengue 2 virus-infected autologous LCL. All the crossreactive clones but one (JK27) lysed dengue 2 virus-infected autologous LCL (Table VII). 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 crossreactive T cells may lyse dengue-infected cells during secondary infections.

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 mAbs
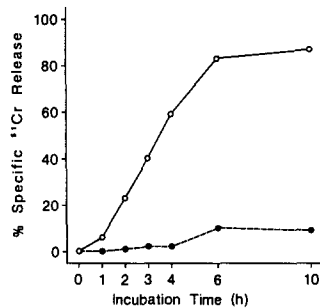

FIGURE 1. Time course of the lysis of dengue 2-infected autologous LCL by JK32. The lysis of dengue 2-infected and uninfected autologous LCL by JK32 was examined at various times. E/T ratio was 2:1. (O) Dengue 2-infected autologous LCL; (●) uninfected autologous LCL.

TABLE VI

Effector-Target Cell Dose-Response Study of the Lysis of Dengue 2 Virus-infected Cells by JK32

| | Percent specific ^{51}Cr release | | |
E/T ratio	Dengue 2-infected autologous LCL	Uninfected autologous LCL	K562
10	60	5	1
3	57	1	1
1	49	0	0
0.3	28	0	0

5×10^3 target cells were incubated with various numbers of effector cells for 4 h.

TABLE VII

*Lysis of Dengue 2 Virus-infected Autologous LCL
by Serotype Cross-reactive T Cell Clones*

			Percent specific ^{51}Cr release		
Serotype	Clones	E/T ratio	Dengue 2-infected autologous LCL	Uninfected autologous LCL	K562
Crossreactive	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
Specific	JK30	3	0	0	0
	JK31	3	0	0	0
	JK37	4	2	2	0

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

to HLA antigens. Anti-HLA DP mAb B7/21.7 inhibited the lysis of dengue 3 antigen-pulsed autologous LCL and dengue 2 virus-infected autologous LCL by JK32, JK34, and JK35. Anti-HLA DQ mAb S3/4 inhibited the lysis of these target cells by JK36 (Table VIII). Anti-HLA class I mAb 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 VIII

*HLA Class II Antigen-restricted Lysis of Target Cells
by Dengue-specific T Cell Clones*

		Percent specific ^{51}Cr release			
Target cells	mAbs	JK32	JK34	JK35	JK36
Dengue 3 antigen-pulsed autologous LCL	–	81	73	22	83
	HLA DR	92	77	30	72
	HLA DP	6	5	2	70
	HLA DQ	78	79	27	9
Dengue 2 virus-infected autologous LCL	–	54	57	22	44
	HLA DR	56	57	19	41
	HLA DP	4	8	2	50
	HLA DQ	45	59	22	8

2.5×10^3 target cells were incubated with 1.25×10^4 effector cells for 4 h in the presence of mAbs, at final dilution of 1:80.
* OKIa1, B7/21.7, and S3/4 were used as anti-HLA DR, anti-HLA DP, and anti-HLA DQ antibodies, respectively.

Discussion

In this paper we report the establishment of dengue antigen–specific human T cell clones and describe the serotype specificity, IFN-γ production, and cytotoxic activity of these T cell clones. Dengue antigen–specific T cell clones were established from the PBL of a donor who had been infected with dengue 3 virus. All of the clones have a CD3$^+$ CD4$^+$ CD8$^-$ phenotype. 8 of 12 clones responded to dengue antigens in a serotype crossreactive fashion. Four of the clones, which we called serotype-specific type, responded predominantly to dengue 3 antigen. Proliferative responses in bulk cultures of the PBMC of this donor were primarily dengue 3 serotype–specific, but also contained serotype crossreactive responses to lower levels. Therefore, the serotype crossreactive responses observed in bulk culture experiments appear to reflect the crossreactive responses detected at the clonal level.

Epidemiological studies have shown that the severe complications of dengue, DHF and DSS, are much more commonly observed during secondary infections than primary infections, and that secondary infections are caused by a different serotype of dengue virus from primary infection (1, 8). Therefore, these results, which demonstrate that most of the dengue-specific T cell clones are serotype crossreactive, support the possibility that dengue crossreactive T cells will be activated during secondary infections with a virus of a heterologous serotype and that these T cells may contribute to the pathogenesis of DHF/DSS.

Cytotoxic functions of these 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 (8). All but one serotype crossreactive clone 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 crossreactive cytotoxic T cell clones are HLA class II restricted. It is known that monocytes are the cells that best support dengue virus infection (20), and monocytic cells with dengue antigens have been observed in DHF/DSS patients (5, 6). 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 crossreactive CTL clones can lyse dengue 2 virus–infected autologous monocytes. Two of the serotype crossreactive clones we have examined to date lysed dengue type 2 virus–infected autologous monocytes, but they did not lyse uninfected monocytes (data not presented).

We have previously reported that CD4$^+$ dengue-specific T lymphocytes proliferate and produce IFN-γ after stimulation with dengue antigens in bulk cultures (9). We have also reported that IFN-γ increases the number of Fcγ receptors on human monocytic cell line and monocytes (21, 22), and that this results in augmented dengue virus infection in the presence of antidengue antibodies (10). Therefore, it was important to learn whether dengue-specific T cell clones produce IFN-γ after stimulation with heterologous dengue virus antigens. Our results demonstrated that most of the serotype crossreactive T cell clones produce IFN-γ after stimulation with dengue antigens of other subtypes, which is consistent with our previous experiments in bulk cultures (9).

Based on these observations we hypothesize roles for dengue-specific T lympho-

cytes in the pathogenesis of DHF/DSS. Dengue serotype crossreactive CD4$^+$ T lymphocytes are activated and produce IFN-γ during secondary infection with a virus strain of a heterologous serotype. IFN-γ increases the number of Fcγ receptors of monocytes, and this augments dengue virus infection of monocytes in the presence of antidengue antibodies. IFN-γ also increases HLA class II antigen expression (23), and activates monocytes to produce inflammatory mediators, which may contribute to the pathogenesis of DHF/DSS. Dengue-infected, IFN-γ-activated monocytes are then lysed by dengue serotype crossreactive CD4$^+$ T lymphocytes, and mediators will be quickly released from these monocytes, which may lead to DHF/DSS.

Although our results support the contribution of dengue-specific CD4$^+$ T lymphocytes in the pathogenesis of DHF/DSS during secondary infections, they do not explain the pathogenesis of DHF/DSS in primary infections. DHF/DSS, which occurs during primary infections, consists of <1% of the cases of DHF/DSS (24), and most of these occur as primary infections of infants from 6 to 12 mo of age born to the dengue antibody–positive mothers (1, 8). Kliks et al., (25) recently reported that levels of maternal antibody correlated with the occurrence of DHF/DSS of the infants during their first year.

We have recently detected CD4$^-$ CD8$^+$ dengue virus–specific T lymphocytes. They lyse dengue virus–infected autologous cells in an HLA class I–restricted fashion (Bukowski, J., I. Kurane, and F. A. Ennis, manuscript in preparation). These results indicate that there are two types of dengue-specific CTL; CD4$^+$ CD8$^-$ HLA class II–restricted CTL described in this report and CD4$^-$ CD8$^+$ HLA class I–restricted CTL. The role of these CTL in the pathogenesis of and in recovery from dengue infections are important subjects to be elucidated.

Mapping of the epitopes that are recognized by these T cell clones is an important task. We have observed that spleen cells from mice immunized with dengue virus proliferate after stimulation with baculovirus-dengue constructs expressing dengue structural and nonstructural proteins (26). Dengue protein constructs prepared by recombinant DNA techniques and synthetic peptides will be used for defining the epitopes that are recognized by these dengue antigen–specific T cell clones. Definition of the epitopes recognized by dengue-specific T cell clones and the MHC haplotypes that restrict these responses will give useful information for attempting development of subunit vaccines against dengue virus infections.

Summary

The severe complications of dengue virus infections, hemorrhagic manifestation and shock, are much more commonly observed during secondary infections caused by a different serotype of dengue virus than that which caused the primary infections. It has been speculated, therefore, that dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are caused by serotype crossreactive immunopathological mechanisms. We analyzed clones of dengue serotype crossreactive T lymphocytes derived from the 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. 12 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 crossreactive, while four other clones responded predomi-

nantly to dengue 3 antigen. These results indicate that the serotype crossreactive dengue-specific T lymphocyte proliferation observed in bulk cultures reflects the cross-reactive responses detected at the clonal level.

Serotype crossreactive clones produced high titers of IFN-γ after stimulation with dengue 3 antigens, and also produced IFN-γ to lower levels after stimulation with dengue 1, 2, and 4 antigens. The crossreactive clones lysed autologous lymphoblastoid cell line (LCL) pulsed with dengue antigens, and the crossreactivity of CTL lysis by T cell clones was consistent with the crossreactivity observed in proliferation assays. Epidemiological studies have shown that secondary infections with dengue 2 virus cause DHF/DSS at a higher rate than the other serotypes. We hypothesized that the lysis of dengue virus–infected cells by CTL may lead to DHF/DSS; therefore, the clones were examined for cytotoxic activity against dengue 2 virus–infected LCL. All but one of the serotype crossreactive clones lysed dengue 2 virus–infected autologous LCL, and they did not lyse uninfected autologous LCL. The lysis of dengue antigen–pulsed or virus–infected LCL by the crossreactive CTL clones that we have examined is restricted by HLA DP or DQ antigens.

These results indicate that primary dengue virus infections induce predominantly crossreactive memory CD4⁺ T lymphocytes. These crossreactive T lymphocytes proliferate and produce IFN-γ after stimulation with a virus strain of another sero-type, and demonstrate crossreactive cyotoxic activity against autologous cells infected with heterologous dengue viruses. Based on these results we hypothesize that dengue serotype crossreactive T lymphocytes may contribute to the pathogenesis of DHF/DSS: (a) by producing IFN-γ, which increases the number of Fcγ receptors and subsequently increases the number of dengue-infected cells by enhanced uptake of dengue virus-antibody complexes; and (b) by lysing dengue virus–infected cells during secondary infections with a virus of the heterologous serotype.

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