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Dengue Virus-Specific Human CD4+ T-Lymphocyte Responses in a Recipient of an Experimental Live-Attenuated Dengue Virus Type 1 Vaccine: Bulk Culture Proliferation, Clonal Analysis, and Precursor Frequency Determination

SHARONE GREEN,[1] ICHIRO KURANE,[1] ROBERT EDELMAN,[2] CAROL O. TACKET,[2] KENNETH H. ECKELS,[3] DAVID W. VAUGHN,[4] CHARLES H. HOKE, JR.,[4] AND FRANCIS A. ENNIS[1]*

Division of Infectious Disease and Immunology, Department of Medicine, University of Massachusetts Medical Center, Worcester, Massachusetts 01655[1]; Center for Vaccine Development, University of Maryland, Baltimore, Maryland 21201[2]; and Department of Virus Diseases[4] and Department of Biologics Research,[3] Walter Reed Army Institute of Research, Washington, D.C. 20307

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We analyzed the CD4+ T-lymphocyte responses to dengue, West Nile, and yellow fever viruses 4 months after immunization of a volunteer with an experimental live-attenuated dengue virus type 1 vaccine (DEN-1 45AZ5). We examined bulk culture proliferation to noninfectious antigens, determined the precursor frequency of specific CD4+ T cells by limiting dilution, and established and analyzed CD4+ T-cell clones. Bulk culture proliferation was predominantly dengue virus type 1 specific with a lesser degree of cross-reactive responses to other dengue virus serotypes, West Nile virus, and yellow fever virus. Precursor frequency determination by limiting dilution in the presence of noninfectious dengue virus antigens revealed a frequency of antigen-reactive cells of 1 in 1,686 peripheral blood mononuclear cells (PBMC) for dengue virus type 1, 1 in 9,870 PBMC for dengue virus type 3, 1 in 14,053 PBMC for dengue virus type 2, and 1 in 17,690 PBMC for dengue virus type 4. Seventeen CD4+ T-cell clones were then established by using infectious dengue virus type 1 as antigen. Two patterns of dengue virus specificity were found in these clones. Thirteen clones were dengue virus type 1 specific, and four clones recognized both dengue virus types 1 and 3. Analysis of human leukocyte antigen (HLA) restriction revealed that five clones are HLA-DRw52 restricted, one clone is HLA-DP3 restricted, and one clone is HLA-DP4 restricted. These results indicate that in this individual, the CD4+ T-lymphocyte responses to immunization with live-attenuated dengue virus type 1 vaccine are predominantly serotype specific and suggest that a multivalent vaccine may be necessary to elicit strong serotype-cross-reactive CD4+ T-lymphocyte responses in such individuals.

Dengue viruses are members of the family *Flaviviridae*; they are transmitted by *Aedes aegypti* mosquitoes. There are an estimated 100 million cases of dengue virus infection in the world each year, occurring predominantly in the tropical and subtropical regions of Central and South America and Southeast Asia (5). The majority of infections are asymptomatic or cause a self-limited illness known as dengue fever. The more severe form of dengue infection, dengue hemorrhagic fever–dengue shock syndrome, is characterized by plasma leakage and may be life threatening (5). Because of the significant morbidity and mortality attributable to dengue viruses, efforts are under way to develop a safe and immunogenic vaccine.

There are four serotypes of dengue virus, types 1, 2, 3, and 4. Immunity to the infecting serotype is believed to be lifelong (5, 6). Following natural infection, cross-reactive antibody responses are elicited; however, protection to heterologous dengue virus serotypes is short lived, on the order of several months (14). Cross-reactive T-cell responses have been observed in humans following natural infection or immunization with live-attenuated dengue virus vaccines (4, 10). Our laboratory has demonstrated dengue virus serotype cross-reactive CD4+ lymphocyte responses in the bulk culture of the peripheral blood mononuclear cells (PBMC) of an individual immunized against dengue virus type 3 (11). Six patterns of dengue virus and flavivirus specificities were noted in CD4+ T-cell clones from this individual: dengue virus type 3 serotype specific, dengue virus subcomplex specific (dengue virus types 1, 2, and 3, and dengue virus types 2, 3, and 4), dengue virus serotype cross-reactive, and two patterns of flavivirus cross-reactivity (8). Cell-mediated immunity is believed to be necessary for the control of virus infections. The level of T-cell recognition of viruses has been studied to a limited degree in the evaluation of the immunogenicity of experimental human immunodeficiency virus vaccines (13). In our study, T-cell memory responses induced in a dengue virus-naive recipient by an experimental live-attenuated dengue virus type 1 vaccine were analyzed at bulk culture, precursor frequency, and clonal levels to determine the immunogenicity of this vaccine. These studies reveal a high CD4+ T-cell precursor frequency to dengue virus type 1 (1 in 1,686) compared with the other three serotypes. Of 17 cytotoxic CD4+ clones, 13 were dengue virus type 1 specific and 4 were dengue virus type 1-dengue virus type 3 cross-reactive.

MATERIALS AND METHODS

Viruses. Dengue virus type 1 (Hawaii strain) and dengue virus type 2 (New Guinea C strain) were provided by Walter E. Brandt, Walter Reed Army Institute of Research. Dengue virus type 3 (CH53489 strain), was provided by Bruce L. Innis, Armed Forces Institute of Medical Science, Bangkok,

* Corresponding author.

Thailand. Dengue virus type 4 (814669 strain) was provided by Jack McCown, Walter Reed Army Institute of Research. Yellow fever virus (YFV) (17D strain) was provided by Jacob J. Schlesinger, University of Rochester School of Medicine and Dentistry. West Nile virus (WNV) (E101 strain) was provided by Margo Brinton, Georgia State University. Viruses were propagated in C6/36 mosquito cells to titers of 10^7 to 10^8 PFU/ml as previously described (9) and frozen at −70°C until use.

Experimental dengue virus type 1 vaccine (DEN-1 45AZ5). The virus seed was originally isolated by Leon Rosen, National Institute of Allergy and Infectious Diseases, from the serum of a child with dengue fever. The virus was isolated and subsequently passaged in diploid fetal rhesus lung (FRhl-2) cell culture. A small plaque was selected and mutagenized with 5-azacytidine. This seed was subsequently prepared by the Salk Institute at the Swiftwater, Pa., facility. The vaccine was administered to two healthy volunteers, who subsequently developed classic dengue fever (12). To further attenuate the strain, the virus was passaged 27 times in primary dog kidney cells (PDK) and vaccines were prepared in FRhl-2 cells from PDK passages 10, 20, and 27. The PDK passage 27 vaccine was given to 10 individuals; only 60% developed neutralizing antibodies (unpublished data).

Preparation of dengue virus antigens. Dengue virus antigens, YFV antigen, and WNV antigen were prepared as previously described (10). Briefly, Vero cells were infected with viruses at a multiplicity of infection of 1 PFU per cell and cultured in minimal essential medium with 2% fetal calf serum (FCS). Cells were removed with cell scrapers when 50% cytopathic effect was observed. They were washed three times in phosphate-buffered saline (PBS) at 4°C, treated with 0.025% glutaraldehyde (Sigma Chemical Co., St. Louis, Mo.) in PBS for 15 min at 4°C, washed three times with PBS, and resuspended in RPMI 1640 medium. Cells were sonicated and centrifuged at 1,500 × *g* for 10 min. The supernatant fluid was collected and used as the virus antigen. Control antigen was prepared with uninfected Vero cells in similar fashion.

Human PBMC. Peripheral blood specimens were obtained from the donor 4 months after vaccination with DEN-1 45AZ5 PDK passage 27. This donor was a 38-year-old white man who had never traveled outside of the United States. His serum did not contain antibody to dengue viruses prior to enrollment in a phase I clinical trial at University of Maryland Center for Vaccine Development. Following immunization, this volunteer experienced a mild dengue illness between days 10 and 19, with rash, malaise, arthralgia, myalgia, eye pain, headache, and nausea but no fever. He did not have detectable viremia but developed immunoglobulin M antibody to dengue virus type 1 and a neutralizing antibody titer of 1:90 at 31 days postvaccination. PBMC were separated by density gradient centrifugation with Ficoll-Hypaque (2). The cells were resuspended at 10^7/ml in RPMI medium with 10% FCS (Sigma) and 10% dimethyl sulfoxide and cryopreserved until use.

Proliferative responses of PBMC. Proliferation assays of PBMC were performed as previously described (10). PBMC (1.5×10^5 to 2.5×10^5) were cultured with viral antigens at various dilutions in 0.2 ml of AIM-V medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% human AB serum (Advanced Biotechnologies, Inc., Columbia, Md.) in 96-well round-bottom microtiter plates (Costar, Cambridge, Mass.) at 37°C for 6 days. The cells were pulsed with 1.25 μCi of tritiated thymidine ([^{3}H]TdR) for 6 h before harvest with a multiharvester (Titertek; Skatron Inc. Sterling, Va.). [^{3}H]TdR incorporation was counted in a liquid scintillation counter (1205 Betaplate; Pharmacia, Wallac Oy, Finland).

Dengue virus-specific precursor frequency by the limiting-dilution method. The limiting-dilution assay was based on the method of Van Oers et al. (17). Thirty replicate wells containing various numbers (range, 625 to 20,000 cells per well) of PBMC were cultured in 0.2 ml of AIM-V containing 10% human AB serum with 1:320 diluted dengue virus antigen, control antigen, or no antigen in the presence of 7×10^4 autologous γ-irradiated (3,500 rads) PBMC and 2 U of recombinant human interleukin-2 (Collaborative Research, Inc., Bedford, Mass.) per ml in 96-well round-bottom microtiter plates. For cord blood PBMC, 18 replicate wells containing various numbers (range, 5,000 to 20,000 cells per well) of PBMC were prepared as above. After 6 days, 50 μl of fresh medium containing 5 U of recombinant human interleukin-2 per ml was added to all wells. The cultures were pulsed 4 to 6 days later with 1.25 μCi of [^{3}H]TdR per well for 8 h and harvested as above. Positive cultures were defined as having levels of [^{3}H]TdR incorporation that were greater than 3 standard deviations above the mean of the 30 replicate unstimulated wells. By this method, the false-positivity rate is limited to less than 0.14% (3).

Establishment of dengue virus-specific T-cell clones by limiting dilution. Dengue virus-specific T-cell clones were established as previously reported (11). PBMC (4×10^6 cells) were cultured with 1:2-diluted dengue virus type 1 in RPMI 1640 medium containing 10% heat-inactivated pooled human AB serum, penicillin-streptomycin, glutamine, and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; Sigma) and placed in 96-well round-bottom microtiter plates. The plates were incubated for 7 days at 37°C. On day 7, the cells were collected and cultured at a concentration of 1, 3, 10, and 100 cells per well in 0.15 ml of AIM-V containing 10% pooled human AB serum, 10% T-cell growth factor (Cellular Products, Inc., Buffalo, N.Y.), 10^5 γ-irradiated (3,500 rads) autologous PBMC, and 1:3-diluted dengue virus type 1. On day 4, 0.05 ml of fresh medium with human AB serum and T-cell growth factor was added. On day 7, 0.1 ml of supernatant was removed and fresh medium was added as above. In addition, 10^5 γ-irradiated (3,500 rads) autologous PBMC and 1:2-diluted dengue virus type 1 were added. On days 14 and 21, growing cells were transferred to 48-well flat-bottom plates (Costar) and cultured with 10^6 γ-irradiated PBMC.

Establishment of lymphoblastoid cell lines. PBMC (2×10^6 cells) were cultured in RPMI 1640 medium containing 10% FCS, penicillin, streptomycin, glutamine, and HEPES in the presence of 1:3-diluted Epstein-Barr virus from an infected marmoset cell line, B95-8 (ATCC), in 24-well flat-bottom plates (Costar) (15).

Preparation of target cells. Lymphoblastoid cells (4×10^5 cells) were cultured for 16 to 20 h in RPMI 1640 medium containing 10% FCS and 1:50 diluted dengue virus, YFV, WNV, or control antigens. The cells were then washed and labeled with ^{51}Cr (Dupont NEN, Boston, Mass.) to be used as target cells.

Cytotoxicity assays. Target cells (4×10^5 cells) were labeled with 0.25 mCi of ^{51}Cr in 0.2 ml RPMI medium plus 10% FCS for 60 min, washed three times with RPMI medium plus 10% FCS, and resuspended at 10^4 cells per ml. Targets were plated at 10^3 per well in 0.1 ml RPMI medium plus 10% FCS in V-bottom microtiter plates (Costar). Effector cells were added at different concentrations in triplicate in 0.1 ml

TABLE 1. Proliferation responses of the PBMC of a dengue virus type 1 vaccine recipient to dengue and flavivirus antigens in bulk culture[a]

Antigen	[^{3}H]TdR incorporation (cpm) at viral antigen dilution of:		
	1:160	1:320	1:640
Dengue virus type 1	41,001	54,265	34,832
Dengue virus type 2	16,387	16,512	7,322
Dengue virus type 3	16,412	17,631	8,030
Dengue virus type 4	7,220	6,501	4,417
WNV	894	13,262	9,043
YFV	14,649	7,642	5,539
Control	161	1,586	1,856

[a] PBMC (2×10^5 cells) were incubated for 6 days in the presence of serial dilutions of dengue virus, flavivirus, and control antigens. Cells were pulsed with 1.25 μCi of [^{3}H]TdR for 6 h, and [^{3}H]TdR incorporation was measured. No antigen control mean is 1,379 cpm.

of RPMI medium plus 10% FCS. The plates were centrifuged at $200 \times g$ for 5 min and then incubated at 37°C for 5 h. Following incubation, the supernatant fluids were harvested and counted in an automatic gamma counter. The percent specific ^{51}Cr release was calculated from the following formula: [(cpm experimental release − cpm spontaneous release)/(cpm maximal release − cpm spontaneous release)] × 100.

Inhibition of lysis of dengue virus type 1 antigen-pulsed target cells by monoclonal antibodies directed against class II human leukocyte antigens (HLA). Monoclonal antibodies B7/21.7, S3/4, and OKIa1 recognize HLA-DP, HLA-DQ, and HLA-DR determinants, respectively. B7/21.7 and S3/4 were kindly provided by Nancy Reinsmoen, University of Minnesota, Minneapolis. OKIa1 was purchased from Ortho Diagnostic Systems, Inc., Raritan, N.J. The 10th International Histocompatibility Workshop lymphoblastoid cell lines (American Society for Histocompatibility and Immunogenetics, Lenexa, Kans.) were used as target cells. A total of 10^3 ^{51}Cr-labeled target cells in 0.05 ml of RPMI medium plus 10% FCS were incubated with 0.05 ml of 1:20-diluted monoclonal antibodies for 30 min. The effector cells were then added in 0.1 ml, and the mixture was incubated for 6 h. The percent specific ^{51}Cr release was determined as described above.

Phenotype analysis. Cell surface antigens CD3, CD4 and CD8 were analyzed by using fluorescein isothiocyanate-conjugated monoclonal antibodies anti-Leu4, anti-Leu2 and anti-Leu3 (Becton Dickinson Co., Mountain View, Calif.), respectively. Briefly, 20 μl of antibody was added to 0.5×10^6 to 1×10^6 cells and incubated for 30 min at 4°C. The cells were then washed twice in cold PBS and analyzed by a fluorescence-activated cell sorter or by fluorescence microscopy.

Statistical analysis. Antigen-reactive cell frequencies were calculated by using a computer-assisted Taswell analysis provided by Richard A. Miller, University of Michigan, Ann Arbor (16).

RESULTS

Proliferative responses of PBMC to noninfectious dengue virus antigens. We first examined the dengue virus serotype specificity and flavivirus specificity of CD4$^+$ memory T cells in bulk culture proliferation assays. PBMC were cultured with antigens of the four dengue virus serotypes, WNV, YFV, or control antigens at 1:160, 1:320 and 1:640 dilutions, and the levels of [^{3}H]TdR incorporation were assessed (Table 1). Dengue virus type 1 antigen induced the highest level of proliferation, and dengue virus types 2, 3, and 4, WNV, and YFV antigens induced proliferation to a lesser extent. Control antigen did not induce a proliferative response. T lymphocytes that respond to these noninfectious dengue virus antigens are predominantly CD4$^+$ (10, 11, 18). These results suggest that dengue virus-specific CD4$^+$ memory T lymphocytes of this donor are predominantly dengue virus type 1 specific and that dengue virus- and flavivirus-cross-reactive CD4$^+$ memory T lymphocytes are present to a lesser degree.

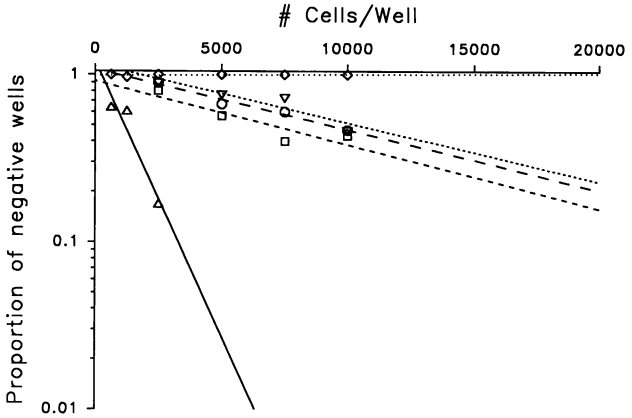

FIG. 1. Precursor frequency analysis of T lymphocytes specific for dengue virus type 1, 2, 3 and 4 antigens. Thirty replicate wells containing various numbers (625 to 10,000 cells per well) of PBMC were cultured in 0.2 ml of AIM-V containing 10% human AB serum with 1:320 diluted dengue virus antigen, control antigen, or no antigen in the presence of 7×10^4 autologous γ-irradiated PBMC and 2 U of recombinant interleukin-2 per ml. A 50-μl volume of media containing 5 U of interleukin-2 per ml was added to all the wells on day 6. [^{3}H]TdR incorporation was examined on day 10. Positive cultures were defined as having levels of [^{3}H]TdR incorporation that were greater than 3 standard deviations above the mean of 30 replicate unstimulated wells. Symbols: ◇, control antigen; △, dengue virus type 1 antigen; ○, dengue virus type 2 antigen; □, dengue virus type 3 antigen; ▽, dengue virus type 4 antigen.

Precursor frequency of memory CD4$^+$ T cells to dengue viruses. To quantitate the level of CD4$^+$ memory T cells induced in this donor by the dengue virus type 1 vaccine, we performed a precursor frequency analysis by limiting dilution in the presence of noninfectious dengue virus antigens as described in Materials and Methods. Results of a representative experiment are shown in Fig. 1. The CD4$^+$ T-cell precursor frequency is highest for dengue virus type 1 (1 in 1,686 PBMC [95% confidence interval, 1,297 to 2,410]) and is followed by those for dengue virus type 3 (1 in 9,870 PBMC [95% confidence interval, 7,781 to 13,492]), dengue virus type 2 (1 in 14,053 PBMC [95% confidence interval, 10,718 to 20,400]), and dengue virus type 4 (1 in 17,690 PBMC [95% confidence interval, 13,204 to 26,792]). As a negative control in a parallel experiment, cord blood PBMC cultured in similar fashion showed no antigen-reactive cells when 18 replicate wells were plated at up to 20,000 PBMC per well (data not shown). These data confirm the results with the bulk cultures, which suggest that the CD4$^+$ memory T lymphocyte induced by the DEN-1 45AZ5 vaccine is predominantly dengue virus type 1 specific.

Virus and serotype specificity of responding cells by estab-

TABLE 2. Dengue serotype and flavivirus specificities of CD4+ cytotoxic T-cell clones[a]

Clone	E:T[b]	% Specific ^{51}Cr release[c] by:							
		D1Ag[d]	D2Ag	D3Ag	D4Ag	WNV[d]	YFV[d]	Cont	NoAg
1	16:1	27	0	0	0	0	0	0	0
3	15:1	30	0	0	0	0	0	0	0
5	12:1	80	0	0	0	0	0	0	0
6	3:1	50	0	0	0	0	0	0	0
7	14:1	74	0	0	0	0	0	0	0
9	14:1	44	0	0	0	0	0	0	0
10	13:1	87	0	28	0	0	0	0	0
13	6:1	44	0	0	0	0	0	0	0
14	16:1	63	0	0	0	0	0	0	0
15	6:1	71	0	64	0	0	0	0	0
16	9:1	27	0	30	0	0	0	0	0
17	6:1	59	0	0	0	0	0	0	0
18	9:1	75	0	66	0	0	0	0	0
20	7:1	70	0	0	0	0	0	0	0
24	15:1	75	0	0	0	0	0	0	0
32	13:1	54	0	0	0	0	0	0	0
33	5:1	22	0	0	0	0	0	0	0

[a] Target cells (1.0×10^3) were incubated with effector cells for 5 h. ^{51}Cr release was calculated as described in Materials and Methods.
[b] E:T, effector-to-target cell ratio.
[c] Underlines indicate significant levels of lysis. Cont, control antigen; NoAg, no antigen.
[d] Dengue virus antigens, WNV antigen, and YFV antigen were prepared as described in Materials and Methods.

TABLE 3. Inhibition of target cell lysis by monoclonal antibodies directed against class II HLA[a]

Clone	% Specific ^{51}Cr release[b] by:				
	Dengue virus type 1 antigen				No Ag
	No antibody	Anti-DP	Anti-DQ	Anti-DR	
7	92	88	81	46[c]	1
9	74	71	67	53	2
10	93	88	84	67	2
17	41	4	44	18	4
20	81	42	97	71	5
24	52	58	63	12	0

[a] A total of 10^3 target cells were incubated with effector cells for 6 h in the presence of monoclonal antibodies at final dilution of 1:80. B7/21.7, S3/4, and OK1a1 were used as anti-HLA-DP, anti-HLA-DQ, and anti-HLA-DR, respectively.
[b] The effector-to-target cell ratio was 8:1 for clone 17; 18:1 for clone 10; 19:1 for clones 7 and 20; and 20:1 for clones 9 and 24.
[c] Underlines indicate significant inhibition of lysis.

lishment of dengue virus-specific CD4+ T-cell clones. To further analyze the dengue virus serotype specificity and flavivirus specificity of CD4+ memory T cells in this donor, we established dengue virus-specific CD4+ CD8− T-cell clones by limiting dilution as described in Materials and Methods. Seventeen CD4+ T-cell clones were established. The flavivirus specificities and dengue virus serotype specificities of the clones were examined in cytotoxic T-lymphocyte assays. The results of these assays are shown in Table 2. Thirteen clones were dengue virus type 1 specific. Four clones recognized both dengue virus type 1 and 3 antigens. Dengue virus types 2 and 4, WNV, and YFV were not recognized by any of these CD4+ T-cell clones. These results are consistent with those obtained in bulk culture proliferation assays and suggest that dengue virus type 1-specific CD4+ T cells were induced predominantly by immunization with this experimental dengue virus type 1 vaccine.

HLA restriction of the lysis of target cells by CD4+ T-cell clones. HLA restriction of the lysis of target cells by six dengue virus-specific CD4+ T-cell clones was first examined by using monoclonal antibodies to HLA class II molecules (Table 3). Monoclonal antibody to HLA-DP inhibited the lysis of target cells by dengue virus type 1-specific clones 17 and 20. Monoclonal antibody to HLA-DQ did not inhibit the lysis of target cells. Monoclonal antibody to HLA-DR inhibited the lysis of dengue virus type 1-specific clones 7, 9, and 24 and dengue virus type 1–dengue virus type 3 cross-reactive clone 10.

To further delineate the HLA restriction of these dengue-specific CD4+ T-cell clones, we analyzed the lysis of dengue virus type 1 antigen-pulsed allogeneic target cells (Table 4). Although the HLA-DP type of the donor is not known, we can infer from the antibody-blocking pattern and from allo-target lysis that the dengue virus type 1-specific clone 17 is HLA-DP4 restricted and that dengue virus type 1-specific clone 20 is HLA-DP3 restricted. Dengue virus type 1-specific clones 7, 9, and 24 and dengue virus type 1–dengue virus type 3 cross-reactive clone 10 appear to be HLA-DRw52 restricted.

DISCUSSION

In this study, we analyzed the dengue virus-specific CD4+ memory T cells in a volunteer who had received an experimental live-attenuated dengue virus type 1 vaccine 4 months earlier. Proliferation to noninfectious dengue virus antigens in bulk culture was predominantly dengue virus type 1 specific, and there was a lower level of serotype-cross-reactive responses. Similar bulk culture responses were noted in another donor whose lymphocytes were examined following natural infection with dengue virus type 1 (11). We also determined the CD4+ precursor frequency and analyzed the responses of cytotoxic CD4+ CD8− T cells at the clonal level. The CD4+ T-cell precursor frequency was highest for dengue virus type 1 and lower for the other dengue virus serotypes. Cord blood PBMC did not react to dengue virus antigens. Of 17 CD4+ T-cell clones established from this donor, 13 were dengue virus type 1 specific and 4 were dengue virus type 1 and dengue virus type 3 cross-reactive. Six clones were analyzed for HLA restriction patterns. One clone was HLA-DP3 restricted, and one was HLA-DP4 restricted. Four clones were HLA-DRw52 restricted. These clones did not lyse all of the HLA-DRw52-matched allogeneic target cells, but this may be due to different subtypes of HLA-DRw52.

These results differ from our previous results, which were obtained by analyzing PBMC of individuals who were infected with dengue virus type 3 or 4 (references 8 and 11 and unpublished observation). In those individuals, bulk culture and clonal analysis revealed more serotype and subcomplex cross-reactivity than was observed in our dengue virus type 1-immune donor. The reasons for this difference are not clear; however, possible explanations are as follows. (i) The differences in the levels of serotype-cross-reactive CD4+ memory T cells after primary infection may be due to the differences of HLA haplotype. T cells of individuals with certain HLA types may dominantly recognize serotype-specific T-cell epitopes, whereas others with different HLA types recognize serotype-cross-reactive epitopes. (ii) Dengue virus type 1 may produce a more serotype-specific CD4+

TABLE 4. Determination of HLA class II restriction of CD4+ T-cell clones with dengue virus type 1 antigen-pulsed allogeneic target cells[a]

Target	HLA class II type[b]				% Specific ^{51}Cr release[c]					
	DR	DR	DP	DQ	7	9	10	17	20	24
Expt 1										
Autologous	1/w6	w52	—[d]	w1	95	85	97	ND[e]	87	83
9004	1	—	4	w5 (w1)	0	2	81	ND	0	0
9011	w15	—	2/4	w6 (w1)	0	0	0	ND	0	0
9038	w12	w52	2	w7	65	32	44	ND	0	0
9049	7	w52	1	w2	0	0	11	ND	0	0
3099	1	—	—	w1	0	2	33	ND	0	0
Expt 2										
Autologous	1/w6	w52	—	w1	100	73	93	78	87	81
9022	w17	w52	3	w2	10	8	3	6	38	29
9052	7	w53	4	w9	0	0	0	32	0	0
9074	9	w53	2/5	w9	0	0	10	5	0	1
9087	3	w52	3/4	w2	3	0	5	25	11	0
3153	w6	—	—	—	6	0	3	9	0	1
JK	2	—	2	w1	0	2	0	0	0	32
Expt 3										
Autologous	1/w6	w52	—	w1	ND	31	ND	15	ND	6
9062	w13	w52	4	w6 (w1)	ND	50	ND	0	ND	0
GM-11	w6	—	—	—	ND	57	ND	0	ND	0
Expt 4										
Autologous	1/w6	w52	—	w1	25	22	38	11	38	19
3103	4	—	2	—	0	0	0	1	0	0
3106	5	—	2	w1	26	10	13	1	0	0
CB	7	w53	4	w2	0	0	0	15	0	0
CP	5/7	w52/w53	4	w2/w3	31	23	14	13	0	0
PG	3/5	w52	—	w2/w3	54	20	39	22	10	0

[a] A total of 1×10^3 targets were incubated with effectors for 5.5 h.
[b] Known HLA loci which match donor HLA are underlined.
[c] The effector-to-target cell ratio was 4:1 for clone 7 (experiments 2 and 4); 5:1 for clone 24 (experiment 3); 8:1 for clones 17 and 20 (experiment 4); 9:1 for clone 9 (experiment 3); 10:1 for clones 10 and 24 (experiment 2); 11:1 for clone 10 (experiment 4); 12:1 for clone 9 (experiment 1); 14:1 for clones 17 (experiment 3), 9 (experiment 4), and 24 (experiment 1); 15:1 for clone 17 (experiment 2); 16:1 for clones 9 and 20 (experiment 2); 17:1 for clone 24 (experiment 4); 19:1 for clone 7 (experiment 1); and 20:1 for clones 10 and 20 (experiment 1). Underlines indicate significant levels of lysis.
[d] —, locus for which HLA type is not known.
[e] ND, not done.

T lymphocyte response than the other serotypes of dengue virus because of the characteristic amino acid sequences of possible CD4+ T-cell epitopes.

Although we used live dengue virus type 1 as the antigen to establish T-cell clones from this donor, only CD4+ T-cell clones were isolated. We have successfully established HLA class I-restricted CD8+ T-cell clones from the PBMC of a dengue virus type 4-immune donor as well as CD4+ T-cell clones with infectious dengue virus as the antigen (unpublished observation). The dengue virus type 1 we used, however, does not infect monocytes-macrophages well compared with the other serotypes of dengue virus. It is possible that a virus that does not infect these cells is better processed as exogenous antigens and stimulates an HLA class II-restricted CD4+ T lymphocyte response and hence production of CD4+ T-cell clones.

The clonal analysis revealed that a majority of the CD4+ T-cell clones were dengue virus type 1 serotype specific, although clonal analysis may not necessarily represent numerically the specificities of the T-cell population in an individual. A number of investigators have studied the virus-specific cytotoxic CD8+ T-lymphocyte precursor frequency (1, 7); however, the CD4+ T-cell precursor frequency has not been well characterized. We performed a CD4+ precursor frequency determination for each dengue virus serotype. The CD4+ T-cell precursor frequency for dengue virus type 1 was quite high (1 in 1,686 PBMC) and was 5 to 10 times greater than the frequency for the other dengue serotypes. Analysis of the CD4+ T-cell clones established from this individual reflects this ratio. None of the clones that we established recognized dengue virus type 2 or 4. According to the precursor frequencies for each serotype of virus, the number of CD4+ T-cell clones specific for dengue virus types 2 and 4 would be expected to be 1 or 2 of the 17 clones. It is possible that the T-cell clones cross-reactive for dengue virus types 2 and 4 would have been apparent if a larger number of clones had been established. Another explanation is that serotype-cross-reactive CD4+ T cells respond to dengue virus type 2, 3, or 4 to a higher degree than to dengue virus type 1. Therefore, although these serotype-cross-reactive CD4+ T cells were detectable at low precursor frequencies by using antigens of other serotypes, they were not established as clones when dengue virus type 1 was used as the stimulating antigen.

The level of the dengue virus type 1-specific memory T cell in this study was high. This may be because the PBMC were obtained from this subject only 4 months after immunization. It will be important to monitor the changes in the levels of memory CD4+ T cells in this subject. We also plan to determine the levels of dengue virus-specific CD8+ T cells

in this individual. Although the relationship between the frequencies of virus-specific memory T cells and the ability to recover from infection is not known, the analysis of the precursor frequencies may provide useful information that could be used to understand the immunogenicity of experimental vaccines and help guide the timing of vaccination in future immunization strategies. The results in this paper suggest that in some individuals, the $CD4^+$ T-cell responses to primary dengue virus type 1 infection are predominantly serotype specific. Thus, if one is trying to induce strong cross-reactive memory T cells in such individuals by immunization, a multivalent dengue virus vaccine should be considered. It is important to determine the percentage of individuals who develop predominantly serotype-specific responses and whether there are correlations between HLA types and the predominant serotype-specific responses. These studies should provide useful information for the development of safe and effective dengue virus vaccines.

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REFERENCES

1. **Borysiewicz, L. K., S. Graham, J. K. Hickling, P. D. Mason, and J. G. P. Sissons.** 1988. Human cytomegalovirus-specific cytotoxic T cells: their precursor frequency and stage specificity. Eur. J. Immunol. **18**:269–275.
2. **Boyum, A.** 1968. Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest. **21**(Suppl. 97):77–89.
3. **Brett, S. J., A. E. Kingston, and M. J. Colston.** 1987. Limiting dilution analysis of the human T cell response to mycobacterial antigens from BCG vaccinated individuals and leprosy patients. Clin. Exp. Immunol. **68**:510–520.
4. **Bukowski, J. F., I. Kurane, C. J. Lai, M. Bray, B. Falgout, and F. A. Ennis.** 1989. Dengue virus-specific cross-reactive $CD8^+$ human cytotoxic T lymphocytes. J. Virol. **63**:5086–5091.
5. **Halstead, S. B.** 1989. Pathogenesis of dengue: challenges to molecular biology. Science **239**:476–481.
6. **Hayes, E. B., and D. J. Gubler.** 1992. Dengue and dengue hemorrhagic fever. Pediatr. Infect. Dis. J. **11**:311–317.
7. **Hoffenbach, A., P. Langlade-Demoyen, G. Dadaglio, E. Vilmer, F. Michel, C. Mayaud, B. Autran, and F. Plata.** 1989. Unusually high frequencies of HIV-specific cytotoxic T lymphocytes in humans. J. Immunol. **142**:452–462.
8. **Kurane, I., M. A. Brinton, A. L. Samson, and F. A. Ennis.** 1991. Dengue virus-specific, human $CD4^+$ $CD8^-$ cytotoxic T-cell clones: multiple patterns of virus cross-reactivity recognized by NS3-specific T-cell clones. J. Virol. **65**:1823–1828.
9. **Kurane, I., D. Hebblewaite, W. E. Brandt, and F. A. Ennis.** 1984. Lysis of dengue virus-infected cells by natural cell-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity. J. Virol. **52**:223–229.
10. **Kurane, I., B. L. Innis, A. Nisalak, C. Hoke, S. Nimmannitya, A. Meager, and F. A. Ennis.** 1989. Human responses to dengue virus antigens. Proliferative responses and interferon gamma production. J. Clin. Invest. **83**:506–513.
11. **Kurane, I., A. Meager, and F. A. Ennis.** 1989. Dengue virus-specific human T cell clones. Serotype crossreactive proliferation, interferon gamma production, and cytotoxic activity. J. Exp. Med. **170**:763–775.
12. **McKee, K. T., Jr., W. H. Bancroft, K. H. Eckels, R. R. Redfield, P. L. Summers, and P. K. Russell.** 1987. Lack of attenuation of a candidate dengue 1 vaccine (45AZ5) in human volunteers. Am. J. Trop. Med. Hyg. **36**:435–442.
13. **Redfield, R. R., D. L. Birx, N. Ketter, E. Tramont, V. Polonis, C. Davis, J. F. Brundage, G. Smith, S. Johnson, A. Fowler, T. Wierzba, A. Shafferman, F. Volvovitz, C. Oster, D. Burke, and the Military Medical Consortium for Applied Retroviral Research.** 1991. A phase I evaluation of the safety and immunogenicity of vaccination with recombinant gp160 in patients with early human immunodeficiency virus infection. N. Engl. J. Med. **324**:1677–1684.
14. **Sabin, A. B.** 1952. Research on dengue during World War II. Am. J. Trop. Med. Hyg. **1**:30–50.
15. **Sly, W. S., G. S. Sekhon, R. Kennett, W. F. Bodmer, and J. Bodmer.** 1976. Permanent lymphoid lines from genetically marked lymphocytes: success with lymphocytes recovered from frozen storage. Tissue Antigens **7**:165–172.
16. **Taswell, C.** 1981. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. J. Immunol. **126**:1614–1619.
17. **Van Oers, M. H. J., J. Pinkster, and W. P. Zeijlemaker.** 1978. Quantification of antigen-reactive cells among human T lymphocytes. Eur. J. Immunol. **8**:477–484.
18. **Zivny, J., I. Kurane, C. O. Tacket, R. Edelman, and F. A. Ennis.** 1993. Dengue virus-specific, human CD4+ cytotoxic T lymphocytes generated in short term culture. Viral Immunol. **6**:143–151.