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Dengue Virus-Specific, Human CD4$^+$ CD8$^-$ Cytotoxic T-Cell Clones: Multiple Patterns of Virus Cross-Reactivity Recognized by NS3-Specific T-Cell Clones

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Thirteen dengue virus-specific, cytotoxic CD4$^+$ CD8$^-$ T-cell clones were established from a donor who was infected with dengue virus type 3. These clones were examined for virus specificity and human leukocyte antigen (HLA) restriction in cytotoxic assays. Six patterns of virus specificities were determined. Two serotype-specific clones recognized only dengue virus type 3. Two dengue virus subcomplex-specific clones recognized dengue virus types 2, 3, and 4, and one subcomplex-specific clone recognized dengue virus types 1, 2, and 3. Four dengue virus serotype-cross-reactive clones recognized dengue virus types 1, 2, 3, and 4. One flavivirus-cross-reactive clone recognized dengue virus types 1, 2, 3, and 4 and West Nile virus (WNV), but did not recognize yellow fever virus (YFV), whereas three flavivirus-cross-reactive clones recognized dengue virus types 1, 2, 3, and 4, WNV, and YFV. HLA restriction in the lysis by these T-cell clones was also heterogeneous. HLA-DP, HLA-DQ, and HLA-DR were used as restriction elements by various T-cell clones. We also examined the recognition of viral nonstructural protein NS3, purified from cells infected with dengue virus type 3 or WNV, by these T-cell clones. One serotype-specific clone, two dengue virus subcomplex-specific clones, and three dengue virus serotype-cross-reactive clones recognized NS3 of dengue virus type 3. One flavivirus-cross-reactive clone recognized NS3 of dengue virus type 3 and WNV. These results indicate that heterogeneous dengue virus-specific CD4$^+$ cytotoxic T cells are stimulated in response to infection with a dengue virus and that a nonstructural protein, NS3, contains multiple dominant T-cell epitopes.

Dengue virus infection induces two types of symptoms: dengue fever and dengue hemorrhagic fever (DHF)-dengue shock syndrome (DSS) (7, 8). Dengue fever is a self-limited febrile disease, whereas 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 (3, 7). The pathogenesis of DHF-DSS is not clearly understood. It has been speculated that augmented dengue virus infection of Fc_γ receptor ($Fc_\gamma R$)-positive monocytes by antibodies to dengue viruses contributes to the pathogenesis (7, 8).

We are 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 (2, 13, 15). CD8$^+$ T cells lyse dengue virus-infected autologous cells in a human leukocyte antigen (HLA) class I-restricted fashion and recognize the viral envelope (E) protein or viral nonstructural proteins (2). CD4$^+$ T cells proliferate and produce gamma interferon (IFN-γ), which up-regulates the expression of $Fc_\gamma RI$ and augments dengue virus infection in the presence of antibody to dengue viruses (13). From 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-γ, which is 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 (14, 16). To further characterize dengue virus-specific CD4$^+$ T cells, we have established CD4$^+$ CD8$^-$ clones from a donor who had been immunized earlier with yellow fever vaccine and was then infected with dengue virus type 3 (15). These T-cell clones produced IFN-γ and lysed dengue antigen-cultured autologous cells. In this paper we report the virus and dengue virus serotype specificity and HLA restriction of these clones. Dengue virus-specific CD4$^+$ T-cell clones are heterogeneous, and there are at least six patterns of virus and dengue virus serotype specificity. HLA-DP, HLA-DQ, and HLA-DR are all used as restriction elements. We are determining the dengue virus proteins recognized by these T-cell clones. Of 12 clones examined to date, 7 recognize the nonstructural protein NS3. These results suggest that these dengue virus serotype-cross-reactive and flavivirus-cross-reactive T cells are activated during secondary infection with a different serotype of dengue virus from that which caused the primary infection and that they may play an important role in the pathogenesis of DHF-DSS.

MATERIALS AND METHODS

Viruses. Dengue virus type 1, Hawaii strain; type 2, New Guinea C strain; type 3, CH53489 strain; and type 4, 814669 strain; yellow fever virus (YFV) (17D strain); and West Nile virus (WNV) (E101 strain) were used in this study. Dengue virus types 1 and 2 were supplied by Walter E. Brandt, Walter Reed Army Institute of Research, Washington, D.C.; type 3 was supplied by Bruce L. Innis, Armed Forces Research Institute of Medical Science, Bangkok, Thailand; and type 4 was supplied by Jack McCown, Walter Reed

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Army Institute of Research. YFV was supplied by Jacob J. Schlesinger, University of Rochester School of Medicine and Dentistry.

Preparation of dengue virus antigens. Dengue virus antigens, YFV antigen, and WNV antigen were prepared by using dengue virus-infected Vero cells as previously reported (13). Vero cells were infected with viruses at an approximate multiplicity of infection of 1 PFU per cell and cultured in minimal essential medium containing 2% fetal calf serum (FCS). When 50% of the monolayer developed cytopathic effects, the cells were removed by using cell scrapers (Costar, Cambridge, Mass.), washed three times with 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 again three times with PBS, and resuspended in RPMI. They were then sonicated with a sonic dismembrator (Fisher Chemical Co.) and centrifuged at 1,500 × *g* for 10 min. The supernatant fluid was collected and used as the virus antigen. Control antigen was prepared in a similar manner by using uninfected Vero cells. Antigen (3 ml) was obtained from 15 75-cm^2 flasks (Costar) of confluent Vero cells.

Preparation of NS1 and NS3 proteins. MK_2 cells were infected with dengue virus type 3 (CH53489) at an approximate multiplicity of infection of 1 PFU per cell and cultured for 2 days in minimal essential medium containing 8% FCS. The medium of one of eight T150 flasks was then replaced with minimal essential medium lacking methionine and containing 0.5% FCS and 2.0 μg of dactinomycin per ml. One hour later, 30 μCi of [^{35}S]methionine per ml was added; the cells were harvested 5 h later.

BHK (strain 2) cells were infected with WNV at a multiplicity of infection of 1, and one flask was incubated with minimal essential medium lacking methionine and with dactinomycin and [^{35}S]methionine added as described above.

At the time of harvest, the infected cells were washed twice with PBS and then scraped into 5 ml of PBS per flask. The cells were pelleted at 350 × *g* for 3 min. The cell pellet was resuspended in 0.24 ml (in three T150 flasks) of 0.1 M Tris (pH 6.8) containing 0.8% sodium dodecyl sulfate (SDS). After cell lysis the SDS concentration was diluted to 0.08%. The cell extract was sheared seven times through a 23-gauge needle and then seven times through a 26-gauge needle. The extract was incubated at 37°C for 2 h with DNase (1.5 μg/ml), and then concentrated gel sample buffer was added to give a final concentration of 0.05 M Tris (pH 6.8), 2% SDS, 10% glycerol, and 0.025% bromophenol blue.

The infected-cell proteins were electrophoresed on multiple SDS–10% polyacrylamide gels. Protein samples were not boiled prior to electrophoresis, so that the NS1 protein dimer would remain intact. The gels were then wrapped in Saran wrap and exposed to X-Omat AR film at 4°C for 5 to 7 days. Viral protein bands (NS1 dimer and NS3) were identified by comparison with lanes containing uninfected control cell extracts and excised. Viral proteins were eluted electrophoretically by using an Elutrap (Schleicher & Schuell, Keene, N.H.) and a buffer containing 25 mM Tris (pH 8.3), 0.1% SDS, and 192 mM glycine. SDS was removed from the eluted protein by dialysis against PBS, and the protein concentration was determined by the bicinchoninic acid (BCA) microprotein assay (Pierce, Rockford, Ill.).

Human PBMC. Peripheral blood specimens were obtained from donor A, who had been immunized with yellow fever vaccine 2 years earlier and was infected with dengue virus type 3 (CH53489) 1 year before the specimens were obtained (9). Peripheral blood mononuclear cells (PBMC) were separated by a Ficoll-Hypaque density gradient centrifugation method (1). Cells were resuspended at 1 × 10^7/ml in RPMI containing 10% FCS (GIBCO Laboratories, Grand Island, N.Y.) and 10% dimethyl sulfoxide (Fisher Scientific Co., Pittsburgh, Pa.) and were cryopreserved until use (11).

Establishment of dengue virus-specific T-cell clones by using a limited dilution method. Dengue virus-specific T-cell clones were established as previously reported (15). PBMC (4 × 10^5) were cultured for 7 days with dengue virus type 2 or 3 antigen at a final dilution of 1:30 in 0.2 ml of RPMI containing 10% human AB serum in 96-well round-bottom plates. On day 7, blast cells were enriched by Ficoll-Hypaque density gradient centrifugation and were cultured at concentrations of 30, 10, 3, 1, and 0.3 cells per well with 10^5 gamma-irradiated (3,000 rads) autologous PBMC in 0.2 ml of RPMI containing 10% human AB serum, 10% T-cell growth factor (Cellular Products, Inc., Buffalo, N.Y.), and dengue virus type 2 or 3 antigen at a final dilution of 1:30 in 96-well round-bottom plates. On day 14, 0.1 ml of medium was removed from each well and 10^5 gamma-irradiated autologous PBMC in 0.1 ml of fresh medium with human AB serum, T-cell growth factor, and dengue virus antigen were added to maintain the same final concentrations described above. On day 21, cells in wells demonstrating growth were transferred to 48-well flat-bottom plates (Costar) and were further cultured with 10^6 gamma-irradiated autologous PBMC in 1 ml of RPMI containing 10% human AB serum, 10% T-cell growth factor, and dengue virus antigen at a dilution of 1:30.

JK21, JK26, JK28, JK32, JK34, JK36 and JK37 were established by using dengue virus type 3 antigen after cloning of one cell per well. JK39, JK41, JK43, JK44, JK46, and JK49 were established by using dengue virus type 2 antigen. JK39 was established after cloning of 0.3 cell per well; JK41 was established after cloning of 3 cells per well; and JK43, JK44, JK46, and JK49 were established after cloning of 10 cells per well.

Preparation of lymphoblastoid cell lines pulsed with dengue virus antigens. Lymphoblastoid cell lines were established by infecting PBMC with Epstein-Barr virus from an infected marmoset cell line supernatant (22). All the transformed cells were cultured in RPMI containing 10% FCS. Epstein-Barr virus was provided by Takeshi Sairenji, University of Massachusetts Medical Center.

A total of 4 × 10^5 lymphoblastoid cell lines were incubated for 24 h with dengue virus antigen, YFV antigen, or WNV antigen at a final dilution of 1:80, with purified dengue virus type 3 NS1, dengue virus type 3 NS3, WNV NS3, or control cell protein at a final concentration of 20 μg/ml in 1 ml of RPMI containing 10% FCS. Cells were washed twice with RPMI–10% FCS, ^{51}Cr labeled, and used as target cells.

Cytotoxicity assays. Target cells (4 × 10^5) were labeled with 0.5 mCi of ^{51}Cr (Na_2CrO_4) (Dupont, NEN, Boston, Mass.) at 37°C for 45 min in 0.2 ml of RPMI–10% FCS. Labeled cells were washed three times and suspended at 2.5 × 10^4/ml in RPMI–10% FCS. Then 2.5 × 10^3 cells in 0.1 ml were added to each well in round-bottom microtiter plates (Linbro Chemical Co., Hamden, Conn.). Various concentrations of effector cells in 0.1 ml of RPMI–10% FCS were added to each well to give the described effector/target ratios. After incubation at 37°C for 6 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 following formula: 100 × (cpm experimental release − cpm spontaneous release)/(cpm maximal release − cpm spontaneous release).

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

Clone	% Specific ^{51}Cr release[b] for:							
	Dengue virus type:				YFV	WNV	Control antigen	No antigen
	1	2	3	4				
Dengue virus serotype specific								
JK21	1	0	20	0	0	0	0	0
JK37	1	0	57	0	0	0	0	0
Dengue virus subcomplex specific								
JK36	0	55	50	15	0	0	0	0
JK46	1	68	67	27	0	0	0	0
JK44	24	65	34	0	0	0	0	0
Dengue virus serotype cross-reactive								
JK32	52	65	66	55	7	8	10	10
JK34	28	54	52	23	0	0	0	0
JK39	39	62	67	31	0	0	0	0
JK41	12	43	39	12	0	0	0	0
Flavivirus cross-reactive								
JK28	24	46	41	25	0	23	0	0
JK26	23	40	41	19	19	55	0	0
JK43	22	31	34	27	37	43	0	0
JK49	34	60	57	26	29	50	0	0

[a] A total of 2.5×10^3 target cells was incubated with effector cells for 6 h.
[b] The percent specific ^{51}Cr release was calculated by using the formula described in Materials and Methods. The effector/target ratio was 3:1 for JK21 and JK43; 4:1 for JK41; 6:1 for JK36, JK37, JK46, JK44, JK32, JK34, JK39, and JK49; 7:1 for JK26; and 12:1 for JK28. Underlines indicate significant levels of lysis.

Antibody blocking of the lysis of dengue virus antigen-cultured target cells. Monoclonal antibodies B7/21.7, S3/4, and OKIa1 recognize HLA-DP, HLA-DQ, and HLA-DR determinants, respectively. Monoclonal antibody W6/32 recognizes a framework determinant of HLA-A, HLA-B, and HLA-C. 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. W6/32 was purchased from Accurate Biochemical Co., Westbury, N.Y. A total of 2.5×10^3 ^{51}Cr-labeled target cells in 0.1 ml were incubated with 0.05 ml of 1:20 diluted monoclonal antibodies for 30 min. The effector cells were then added in 0.05 ml, and the mixture was incubated for 6 h. The percent specific ^{51}Cr release was determined as described above.

Phenotypic analysis. Anti-Leu-2 (4), anti-Leu-3 (4), and anti-Leu-4 (18) antibodies were used as antibodies to CD8, CD4, and CD3, respectively. Anti-Leu-2, anti-Leu-3, and anti-Leu-4 antibodies were purchased from Becton Dickinson Co., Mountain View, Calif. Clones were typed with fluorescein isothiocyanate-conjugated monoclonal antibodies by direct immunofluorescence methods as described earlier (12). The percentage of antigen-positive cells was determined by using a fluorescence-activated cell sorter (no. 440; Becton Dickinson Co.).

RESULTS

Virus and dengue virus serotype specificity of $CD4^+$ T-cell clones. Dengue virus-specific T-cell clones were established from lymphocytes of donor A by using limiting-dilution methods as described above. Seven clones were established by using dengue virus type 3 antigen (15), and six clones were established by using dengue virus type 2 antigen. Phenotypic analyses with monoclonal antibodies showed that all the clones have $CD3^+$ $CD4^+$ $CD8^-$ phenotypes.

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

JK28 lysed target cells cultured with dengue virus antigen of four serotypes and WNV antigen, but did not lyse target cells cultured with YFV antigen. JK26, JK43, and JK49 lysed target cells cultured with dengue virus antigen of four serotypes and YFV and WNV antigens. Therefore, these clones are flavivirus cross-reactive.

These results indicate that dengue virus-specific $CD4^+$ T cells are heterogeneous in virus and dengue virus serotype specificity and that there are at least six patterns of specificity.

HLA restriction in the lysis of target cells by $CD4^+$ T-cell clones. HLA restriction of the lysis of target cells by dengue virus-specific $CD4^+$ T-cell clones were examined by using monoclonal antibodies to HLA molecules (Table 2). Monoclonal antibody to HLA-DP inhibited the lysis of target cells by dengue virus type 3-specific clone JK37; dengue virus serotype-cross-reactive clones JK32, JK34, JK39, and JK41; and flavivirus-cross-reactive clones JK26, JK28, and JK49.

TABLE 2. HLA restriction of lysis of dengue virus type 3 antigen-cultured target cells by CD4+ T-cell clones[a]

Clone	% Specific ^{51}Cr release for[b]:			
	No antigen	Anti-HLA-DP	Anti-HLA-DQ	Anti-HLA-DR
Dengue virus serotype specific				
JK21	22	21	26	6
JK37	55	5	48	26
Dengue virus subcomplex specific				
JK36	25	26	4	20
JK46	45	47	8	41
JK44	51	46	54	14
Dengue virus serotype cross-reactive				
JK32	83	7	80	71
JK34	87	1	83	77
JK39	46	1	48	45
JK41	56	7	47	40
Flavivirus cross-reactive				
JK28	91	11	78	77
JK26	64	1	64	69
JK43	56	49	55	59
JK49	53	1	41	57

[a] A total of 2.5×10^3 target cells were incubated with effector cells for 6 h in the presence of monoclonal antibodies at final dilution of 1:80.
[b] B7/21.7, S3/4, and OKIa1 were used as anti-HLA-DP, anti-HLA-DQ, and anti-HLA-DR, respectively. The percent specific ^{51}Cr release was calculated by using the formula described in Materials and Methods. The effector/target ratio was 4:1 for JK43; 5:1 for JK21, JK39, and JK46; 6:1 for JK37, JK26, JK32, JK44, and JK49; 7:1 for JK36; 11:1 for JK28; 14:1 for JK34; and 15:1 for JK41. Underlines indicate significant inhibition by each antibody.

Monoclonal antibody to HLA-DQ inhibited the lysis of target cells by dengue virus subcomplex-specific clones JK36 and JK46. Monoclonal antibody to HLA-DR inhibited the lysis of target cells by dengue virus type 3-specific clone JK21 and subcomplex-specific clone JK44. Interestingly, the lysis of target cells by flavivirus-cross-reactive clone JK43 was not inhibited by any of the three monoclonal antibodies to HLA class II or by an antibody to HLA class I. However, the lysis by JK43 was inhibited by a mixture of anti-HLA-DP, HLA-DQ, and HLA-DR antibodies and by an antibody to CD3 (data not presented).

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

Recognition of NS3 by dengue virus-specific T-cell clones. We have previously reported that NS3 induces high levels of proliferation responses of donor A PBMC in bulk cultures (17). We next tried to determine whether CD4+ T-cell clones recognize purified NS3 protein (Table 3). The dengue virus type 3-specific clone JK37, the subcomplex-specific clones JK36 and JK46, and the serotype-cross-reactive clones JK32, JK34, and JK39 lysed target cells cultured with NS3 protein purified from dengue virus type 3-infected cells, but did not lyse target cells cultured with NS1 protein purified from dengue virus type 3-infected cells or NS3 from WNV-infected cells. The flavivirus-cross-reactive clone JK43 lysed target cells cultured with NS3 obtained from dengue virus type 3-infected cells or from WNV-infected cells, but did not lyse target cells cultured with NS1 obtained from dengue virus type 3-infected cells.

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

DISCUSSION

In this article we have reported that (i) Dengue virus-specific, human CD4+ CD8− T-cell clones are heterogeneous with at least six patterns of virus and serotype specificities; (ii) HLA restriction of cytotoxicity by dengue virus-specific CD4+ T-cell clones is also heterogeneous with HLA-DP, HLA-DQ, and HLA-DR, each being used as restriction elements by individual CTL clones; and (iii) 7 of the 12 clones examined recognize epitopes on the NS3 protein. Table 4 gives a summary of these results.

We have previously reported that most of these T-cell clones produce IFN-γ after stimulation with dengue virus antigen (15) and that IFN-γ, which up-regulates $Fc_\gamma R$, augments dengue virus infection of $Fc_\gamma R$-positive monocytic cells in the presence of dengue virus antibodies. From these observations we hypothesized that CD4+ T cells may contribute to the pathogenesis of DHF-DSS by producing IFN-γ and by lysing dengue virus-infected monocytes (14, 16). Epidemiological studies have shown that DHF-DSS is much more commonly observed during a secondary infection with a different serotype from that which caused the primary infection (3, 7). Although the specificities of the clones may not accurately reflect the specificities of the in vivo, uncloned, dengue virus-specific CTL, the identification of serotype-cross-reactive and flavivirus-cross-reactive CD4+ T cells supports the possibility that such T cells are activated during secondary infection with a dengue virus of a heterologous serotype and that these T cells contribute to the pathogenesis of DHF-DSS.

Of 13 clones, 7 were found to recognize the NS3 protein. This result is consistent with our previous observation that purified NS3 induced a high level of proliferation of PBMC from donor A in bulk cultures (17). The clones which

TABLE 3. Recognition of NS3 protein by dengue virus-specific CD4+ T-cell clones[a]

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

[a] A total of 2.5×10^3 target cells were incubated with effector cells for 6 h.
[b] Autologous Epstein-Barr virus-transformed cells were cultured with dengue virus type 3 NS1, dengue virus type 3 NS3, WNV NS3, and control cell protein at final concentration of 20 μg/ml with and dengue virus type 3 and WNV antigens at 1:80 for 24 h prior to addition of effector T cells. The percent specific ^{51}Cr release was calculated by using the formula described in Materials and Methods. The effector/target ratio was 4:1 for JK32; 6:1 for JK26, JK37, JK39, and JK44; 7:1 for JK46; 8:1 for JK41 and JK43; 4:1 for JK49; 10:1 for JK28 and JK34; and 11:1 for JK36. Underlines indicate significant levels of lysis.
[c] ND, Not done.

recognize NS3 are heterogeneous. They include a dengue virus type 3-specific clone (JK37), subcomplex-specific clones (JK36 and JK46), dengue virus serotype-cross-reactive clones (JK32, JK34, and JK39) and a flavivirus-cross-reactive clone (JK43). It was surprising to find that NS3 is so immunogenic since it is one of the seven flavivirus nonstructural proteins and is tightly attached to endoplasmic reticular membranes in infected cells (6). NS3 is composed of 618 amino acids (19) and has a mass of 67 kDa. It is hydrophilic and has a net positive charge. It contains conserved epitopes characteristic of helicases (5), and it has also been reported to have proteinase activity (20). The two largest flavivirus nonstructural proteins, NS5 (96 kDa), which is thought to be the viral replicase, and NS3, are the most highly conserved among the flaviviruses. There is a 75% homology between the amino acid sequence of the NS3 of dengue virus type 4 (the 814669 strain) and that of dengue virus type 2 (New Guinea C strain) (10), 62% homology between the NS3s of dengue virus type 4 and WNV, and 51% homology between the NS3s of dengue virus type 4 and YFV (19). The presence of dengue virus subcomplex-specific, serotype-cross-reactive, and flavivirus-cross-reactive T-cell clones is consistent with the high level of amino acid conservation observed for NS3.

TABLE 4. Summary of dengue virus-specific CD4+ CD8− T-cell clones

Virus and serotype specificity[a]	Clone	HLA restriction	Protein recognized[b]	
			NS3	NS1
Serotype specific				
D3	JK21	DR	NT	NT
	JK37	DP	+	−
Subcomplex specific				
D2, D3, D4	JK36	DQ	+	−
	JK46	DQ	+	−
D1, D2, D3	JK44	DR	−	−
Dengue virus serotype cross-reactive				
D1, D2, D3, D4	JK32	DP	+	−
	JK34	DP	+	−
	JK39	DP	+	−
	JK41	DP	−	−
Flavivirus cross-reactive				
D1, D2, D3, D4, WNV	JK28	DP	−	−
D1, D2, D3, D4, YFV, WNV	JK26	DP	−	−
	JK43	?[c]	+	−
	JK49	DP	−	−

[a] D1, D2, D3, D4, Dengue virus types 1, 2, 3, and 4, respectively.
[b] +, Recognition; −, no recognition; NT, not tested.
[c] ?, Undetermined.

The observation that T-cell clones with four different serotype specificities recognize NS3 strongly suggests that NS3 has at least four epitopes recognized by dengue virus-specific CD4+ T cells. It will be important to localize these multiple T-cell epitopes on NS3. This will be performed by using recombinant vaccinia viruses containing truncated dengue virus NS3 genome and with synthetic peptides. It is clear that NS3 is not the only protein which contains CD4+ T-cell epitopes, since some of the CTL clones do not recognize either NS3 or NS1. Therefore, identification of other proteins which contain epitopes recognized by these T-cell clones also remains to be done.

We have observed that the viral proteins recognized by dengue virus-specific murine CTL vary with the mouse strains (21). Therefore, it is likely that dengue virus-specific T cells from other donors who possess different HLA alleles from those of donor A will recognize different epitopes on dengue virus proteins. To gain a complete understanding of

the T-cell response to dengue viruses in humans, it will be necessary to define the epitopes recognized by dengue virus-specific T cells from individuals with different major histocompatibility complex antigens as well as from individuals infected with various dengue viruses.

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