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Establishment and Characterization of Japanese Encephalitis Virus-Specific, Human $CD4^+$ T-Cell Clones: Flavivirus Cross-Reactivity, Protein Recognition, and Cytotoxic Activity

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We analyzed the $CD4^+$ T-lymphocyte responses of two donors who had received Japanese encephalitis virus (JEV) vaccine 6 or 12 months earlier. Bulk culture proliferation assays showed that peripheral blood mononuclear cells (PBMC) responded to JEV antigens (Ag) but also responded at lower levels to West Nile virus (WNV) and dengue virus type 1, 2, and 4 (D1V, D2V, and D4V, respectively) Ag. Five JEV-specific $CD4^+$ human T-cell clones and one subclone were established from PBMC of these two donors. Two clones responded to WNV Ag as well as to JEV Ag, whereas the others responded only to JEV Ag. Three of five $CD4^+$ T-cell clones had JEV-specific cytotoxic activity and recognized E protein. The HLA restriction of the JEV-specific T-cell clones was examined. Three clones were HLA-DR4 restricted, one was HLA-DQ3 restricted, and the HLA restriction of one clone was not determined. T-cell receptor analysis showed that these clones expressed different T-cell receptors, suggesting that they originated from different T lymphocytes. These results indicate that JEV vaccine induces JEV-specific and flavivirus-cross-reactive $CD4^+$ T lymphocytes and that these T lymphocytes recognize E protein. The functions and HLA restriction patterns of these T lymphocytes are, however, heterogeneous.

Japanese encephalitis virus (JEV) is a member of the *Flaviviridae* and is widely distributed in Japan, China, Taiwan, Korea, Philippines, far eastern Russia, and southeastern Asia (10, 19, 22). The clinical features are manifested as a febrile headache syndrome, aseptic meningitis, or encephalitis (3, 5). Clinically overt JEV infection causes impaired consciousness and paralysis of extremities. Death occurs on days 5 to 9 or during a more protracted course with cardiopulmonary implications. The fatality rate is 5 to 40% (19). Neuropsychiatric sequelae occur in survivors and are particularly severe in children (17).

The JEV vaccine which is currently available in Japan is a formalin-inactivated virion preparation purified from JEV-infected mouse brains (21). This vaccine was demonstrated to be safe and efficacious against JEV infections (9). However, there is some concern about this JEV vaccine. Preparation of the vaccine from infected mouse brains requires biosafety precautions. The vaccine is too expensive for use in developing countries. Furthermore, it is possible that the vaccine may still contain a tiny amount of mouse brain components. Thus, the development of a new JEV vaccine is a project to be addressed.

A new JEV vaccine should contain epitopes which induce strong protective immunity against JEV infection. It is generally accepted that neutralizing antibody plays an important role in protection and recovery from JEV infection; however, the role of T-cell-mediated immunity is not well understood. It was reported that helper T lymphocytes were predominant in perivascular infiltrates and that cytotoxic T lymphocytes (CTLs) represented a rather minor population (15–17, 19, 20).

In this paper, we report that peripheral blood mononuclear cells (PBMC) obtained from donors who received JEV vaccine responded to West Nile virus (WNV) and dengue viruses as well as to JEV in bulk cultures. We established and characterized JEV-specific human $CD4^+$ T-cell clones. We analyzed the T-cell clones, focusing on cross-reactivity to other flaviviruses, cytotoxic activity, recognition of viral protein, and HLA restriction. Two T-cell clones were cross-reactive to WNV, while the other clones responded only to JEV. Some clones were cytotoxic for autologous target cells expressing JEV E protein. This is the first report of JEV-specific human $CD4^+$ T-cell clones.

MATERIALS AND METHODS

Virus. JEV (Nakayama strain) was provided by Eiji Konishi, Kobe University School of Medicine. JEV was propagated in C6/36 cells as previously described (13). Briefly, C6/36 monolayers were infected with JEV at a multiplicity of infection (MOI) of 5 PFU/cell and were incubated at 28°C in minimal essential medium containing 2% fetal calf serum (FCS) for 3 to 4 days. The supernatants were collected and stored at −80°C until use. The viral titers of the supernatants were approximately 1.2×10^8 PFU/ml in plaque assays on Vero cells. Recombinant vaccinia viruses vP829, vP658, vP555, and vP410 were provided by Virogenetics, Troy, N.Y. vP829, vP658, and vP555 carried the prM and E genes of the Nakayama strain of JEV, the E and NS1 genes, and the prM, E, and NS1 genes, respectively. vP410 did not contain any JEV genes.

Preparation of flavivirus Ag. JEV antigens (Ag) were prepared from JEV-infected Vero cells as previously described (12). Briefly, Vero cells were infected at an MOI of 5 PFU/cell and incubated at 37°C in minimal essential medium containing 2% FCS until 50% of the cells displayed a cytopathic effect. Cells were harvested by scraping, washed in phosphate-buffered saline (PBS), fixed with 0.025% glutaraldehyde in PBS for 15 min on ice, washed three times in PBS, and resuspended at 3×10^8 cells/ml in RPMI 1640. The fixed cells were sonicated on ice with Ultra S homogenizer VP-15S (Taitec, Saitama, Japan) and centrifuged at $1,500 \times g$ for 10 min at 4°C. The supernatants were collected, divided into aliquots, and frozen at −80°C. Control Ag was prepared similarly from uninfected Vero cell monolayers. Dengue virus type 1, 2, 3, and 4 (D1V, D2V, D3V, and D4V, respectively) and WNV Ag were provided by Francis A. Ennis, University of Massachusetts Medical Center.

Human PBMC. Peripheral blood specimens were obtained from two healthy Japanese adults, donors A and C, who were immunized with JEV (Beijing strain) vaccine 6 to 18 months earlier. These donors had also been immunized with JEV vaccine a few decades earlier. PBMC were purified by Ficoll-Hypaque density gradient centrifugation (1). Cells were resuspended at 10^7/ml in RPMI 1640 with 10% FCS and 10% dimethyl sulfoxide and cryopreserved until use. The HLA

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types of donor A were A2,A24; B38-B59; CW1,CW7; and DRB1*0405,DRB1*0803, DQB1*0301,DQB1*0401, and DPB1*0402,DPB1*1401, which were considered to be DR4,DR8, DQ3,DQ4, and DPw4, respectively. The HLA types of donor C were A24,A33; B52,B35; CW3; and DRB1*0405,DRB1*1502, DQB1*0401,DQB1*0601, and DPB1*0201,DPB1*0901, which were considered to be DR4,DR15, DQ4,DQ6, and DPw2, respectively. The HLA types of donor B were A24,A33; B44; DQ1,DQ3; and DR2,DR5.

Proliferative responses of PBMC in bulk cultures. Proliferation assays for PBMC were performed as previously described (12). PBMC (1×10^5 to 2×10^5) were cultured with 1:100- to 1:200-diluted Ag in 0.2 ml of AIM-V medium (GIBCO) containing 10% heat-inactivated human AB serum (Advanced Biotechnology, Inc., Columbia, Md.) in 96-well V-bottom microtiter plates (Coster, Cambridge, Mass.) at 37°C for 7 days. The cells were pulsed with 1 μCi of tritiated thymidine ([^{3}H]thymidine) for 18 h before harvest. They were harvested with a multiharvester (Skatron Inc., Sterling, Va.), and [^{3}H]thymidine incorporation was counted in a liquid scintillation counter.

Establishment of JEV-specific T-cell clones by limiting dilution. JEV-specific T-cell clones were established by limiting dilution as previously described (6, 23). PBMC were stimulated with JEV Ag for 7 days. T-cell blasts isolated from stimulated PBMC were cloned by limiting dilution. T cells from donor A were cloned at 1 cell per well and T cells from donor C were cloned at 10 cells per well and then recloned at 0.3 cell per well in 0.2 ml of AIM-V medium containing 10% FCS and 20 U of recombinant interleukin 2 (IL-2) per ml in 96-well round-bottom microtiter plates. Mitomycin C (0.05 mg/ml)-treated or gamma-irradiated (3,500 rads) autologous PBMC (10^5) and 1:200- to 1:400-diluted JEV Ag were added to each well. Every 3 to 4 days, 0.1 ml of medium was removed from each well and replaced with AIM-V medium containing 10% FCS and 20 U of IL-2 per ml. On days 10 to 14, growing cells were tested for Ag specificity. Clones showing a stimulation index (SI) of greater than 2.0 were considered to be JEV specific and were expanded for further studies. The cloning efficiency was less than 1%. Clones were restimulated with 1:200- to 1:400-diluted JEV Ag in the presence of gamma-irradiated or mitomycin C-treated PBMC (10^6) in 1.0 ml of AIM-V medium containing 10% FCS and 20 U of IL-2 per ml in 48-well plates (Iwaki Glass, Tokyo, Japan).

Immunofluorescence staining. A total of 6×10^4 to 12×10^4 cells were stained with fluorescein isothiocyanate-labeled anti-CD3, anti-CD4, and anti-CD8 (DAKO A/S, Glostrup, Denmark) for 30 min on ice and washed in PBS containing 2% FCS. The cells were resuspended in 50% glycerol in PBS and examined with a fluorescence microscope.

Proliferation assays of T-cell clones. T-cell clones (1×10^4 to 2×10^4 cells/well) were cultured with gamma-irradiated or mitomycin C-treated PBMC (1×10^5 to 2×10^5 cells/well) in the presence or absence of Ag in 96-well V-bottom microtiter plates. After 48 h of culturing, [^{3}H]thymidine (1 μCi/well) was added, cells were harvested 18 h later, and [^{3}H]thymidine uptake was quantitated with a scintillation counter. The SI was calculated with the formula counts per minute induced by stimulation with viral Ag divided by counts per minute induced by stimulation with control Ag. Proliferation was considered to be significant when (i) the SI was greater than 2.0 and (ii) [^{3}H]thymidine incorporation was greater than 1,000 cpm. In some experiments, optimal concentrations of monoclonal antibodies to HLA-DQ or HLA-DR (Cosmo Bio Co., Ltd., Tokyo, Japan) or mouse immunoglobulin G were added to the cultures for determining HLA restriction.

Establishment of BLCL. B-lymphoblastoid cell lines (BLCL) were established as previously reported (14). PBMC (1×10^6 to 2×10^6) were cultured with 1:3-diluted supernatants of B95-8 cells in RPMI 1640 containing 20% FCS, penicillin, and streptomycin. B95-8 cells were provided by Takeshi Sairenji, Tottori University School of Medicine.

Preparation of target cells. A total of 1×10^5 to 1.5×10^5 cells of Epstein-Barr virus-transformed BLCL were washed once in RPMI 1640 containing 2% FCS and infected with JEV-recombinant vaccinia viruses at an MOI of 10 PFU/cell in RPMI 1640 containing 2% FCS at 37°C for 2 h. Cells were cultured in 2 ml of RPMI 1640 containing 10% FCS in 24-well plates for 16 to 20 h. Infected BLCL were washed and labeled with 0.25 mCi of ^{51}Cr in 0.1 ml of RPMI 1640 containing 10% FCS at 37°C for 1 h. After being labeled, cells were washed four times in RPMI 1640 containing 10% FCS to remove unincorporated ^{51}Cr. The cells were counted and diluted to 10^4 cells per ml for cytotoxicity assays.

Cytotoxicity assays. Assays were performed with 96-well V-bottom plates as previously described (2, 14). Effector cells in 0.1 ml of RPMI 1640 containing 10% FCS were added to 10^3 ^{51}Cr-labeled target cells at effector cell/target cell (E/T) ratios of 10:1 to 20:1. Plates were incubated at 37°C for 5 h. Supernatant fluids were harvested, and ^{51}Cr content was measured with an automatic gamma counter (Auto Well Gamma System ARC-300; Tokyo, Aloka, Japan). The percent specific ^{51}Cr release was calculated with the formula [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100 (release measured in counts per minute). The assays were done in triplicate, and the average for triplicate wells was calculated.

Analysis of TCR V-gene usage and sequencing of complementarity determining region 3 (CDR3). T-cell receptor (TCR) V-gene usage by T-cell clones was analyzed with an adaptor ligation PCR-based microplate hybridization assay as previously reported (18). Briefly, 43 TCR-α V-gene (TCRAV)- and 38 TCR-β V-gene (TCRBV)-specific probes were immobilized in water-soluble carbodiimide in microplate wells. After hybridization of 5′-biotinylated PCR products was carried out as previously reported (18), quantitative enzyme-linked immunosorbent assays were carried out and followed by automated colorimetric reading.

After the TCRAV and TCRBV repertoires of T-cell clones were determined, PCR was performed for 20 cycles with 50-μl volumes and with defined TCRAV- or TCRBV-specific primers and primer CA4 or CB4 (18). The PCR products were purified with a QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany). The purified PCR products were dissolved in 50 μl of distilled water, and the sequences were analyzed by cycle sequencing with Sequence PRO (Toyobo, Osaka, Japan).

TABLE 1. Proliferation of PBMC from donors A and C in response to serially diluted JEV Ag in bulk cultures

PBMC donor	Antigen dilution	[^{3}H]thymidine incorporation (cpm) induced by the following Ag: JEV	Control
A[a]	1:100	11,622	3,462
	1:200	10,943	158
	1:400	8,973	116
	1:800	4,387	515
	1:1,600	1,593	182
C[b]	1:80	13,082	1,032
	1:160	8,595	1,650
	1:320	7,644	2,990
	1:640	2,453	2,027
	1:1,280	3,203	1,488

[a] PBMC (1.5×10^5 cells) from donor A were cultured with serially diluted JEV Ag or control Ag for 7 days. [^{3}H]thymidine incorporation without Ag was 408 cpm. The data shown are the averages for three wells.

[b] PBMC (2.0×10^5 cells) from donor C were cultured with serially diluted JEV Ag or control Ag for 7 days. [^{3}H]thymidine incorporation without Ag was 1,856 cpm. The data shown are the averages for three or four wells.

RESULTS

Proliferative responses of PBMC to JEV Ag in bulk cultures. We first examined PBMC obtained from donors A and C for proliferative responses to JEV Ag. These donors had received JEV vaccine 6 to 12 months earlier. PBMC from donors A and C showed significant proliferative responses after stimulation with JEV Ag, and there was a dose-response relationship (Table 1). High levels of proliferation were detected when PBMC from donor A were cultured with 1:100- to 1:400-diluted JEV Ag and when PBMC from donor B were cultured with 1:80- to 1:320-diluted JEV Ag. Thus, we used JEV Ag at 1:100 to 1:200 dilutions in the next proliferation assays.

Proliferative responses of PBMC to other flavivirus Ag in bulk cultures. PBMC from donor A were cultured with flavivirus Ag at final dilutions of 1:100 and 1:200 for 7 days, and [^{3}H]thymidine incorporation was measured. These PBMC proliferated after stimulation with JEV Ag and proliferated to lower levels after stimulation with D1V, D2V, D4V, and WNV Ag (Table 2). PBMC from donor C proliferated after stimulation with D1V, D2V, D3V, D4V, and WNV Ag (data not shown). These donors had never been to the areas where dengue viruses and WNV were prevalent. The results therefore suggest that memory T lymphocytes induced by immunization with JEV vaccine are predominantly JEV specific but also include T cells cross-reactive to other flaviviruses.

Proliferative responses of CD4$^+$ T-cell clones to flavivirus Ag. We established four CD4$^+$ T-cell clones, A3, A19, A23, and A26, from donor A PBMC and one JEV-specific CD4$^+$ T-cell clone, C2, and its subclone, C2-16, from donor C PBMC by limiting dilution as described in Materials and Methods. These clones were selected based on significant levels of proliferative responses to JEV Ag. All of the clones had a phe-

TABLE 2. Proliferation of PBMC from donor A in response to JEV Ag and other flavivirus Ag[a]

Ag	[^{3}H]thymidine incorporation, in cpm (SI), at an Ag dilution of:	
	1:100	1:200
JEV	61,748 (18.0)	43,157 (16.0)
D1V	6,562 (2.0)	15,214 (5.6)
D2V	17,966 (5.3)	14,316 (5.3)
D3V	4,762 (1.4)	2,728 (1.0)
D4V	1,198 (0.4)	16,615 (6.1)
WNV	7,285 (2.2)	21,601 (8.0)
Control	3,363	2,705

[a] PBMC (2×10^5 cells) were cultured with JEV, dengue virus, WNV, or control Ag at final dilutions of 1:100 and 1:200 for 7 days. Wells were pulsed with 1 μCi of [^{3}H]thymidine for 18 h, and [^{3}H]thymidine incorporation was measured. [^{3}H]thymidine incorporation without Ag was 1,720 cpm. The data shown are the averages for three wells. SIs were calculated with the following formula: mean counts per minute of cultures after stimulation with JEV, dengue virus, or WNV Ag/mean counts per minute of cultures after stimulation with control Ag.

notype of CD3$^+$ CD4$^+$ CD8$^-$, as determined by immunofluorescence staining (data not shown).

We examined these T-cell clones for cross-reactive responses to WNV, D1V, D2V, D3V, and D4V Ag. These CD4$^+$ T-cell clones were divided into two groups based on their responses to flavivirus Ag (Table 3). (i) A19, A23, C2, and C2-16 responded to JEV Ag but not to any other flavivirus Ag tested. (ii) A3 and A26 responded to JEV and WNV Ag but not to dengue virus Ag. Clone A3 responded at lower levels to WNV Ag than to JEV Ag, while clone A26 responded at similar or higher levels to WNV Ag than to JEV Ag. These results suggest that JEV-responsive CD4$^+$ T-cell clones have heterogeneous flavivirus cross-reactivities.

Lysis of autologous target cells expressing JEV proteins by CD4$^+$ T-cell clones. The CD4$^+$ T-cell clones were examined for JEV-specific cytotoxic activities. Autologous BLCL were infected with vP829 (a recombinant vaccinia virus containing the prM and E genes of JEV) and were used as target cells in CTL assays. The JEV-responsive T-cell clones A19, A26, C2, and C2-16 lysed vP829-infected target cells, whereas A3 and A23 did not (Table 4).

Recognition of JEV E protein by CD4$^+$ T-cell clones. Lysis of vP829-infected BLCL by the JEV-specific and JEV- and WNV-cross-reactive CTL clones suggests that these CTL clones recognize either prM or E protein of JEV. We examined the lysis of target cells infected with vP829, vP555 (a recombinant vaccinia virus containing the prM, E, and NS1 genes of JEV), or vP658 (a recombinant vaccinia virus containing the E and NS1 genes of JEV) in order to determine which viral protein was

TABLE 4. Lysis of vP829-infected target cells by JEV-responsive T-cell clones[a]

Clone	% Specific ^{51}Cr release from autologous target cells infected with:	
	vP829	vP410
A3	4	1
A19	76	25
A23	6	1
A26	59	0
C2	76	3
C2-16	48	0

[a] Autologous target cells (10^3) infected with vP829 (vaccinia virus recombinant containing the prM and E genes of JEV) or vP410 (control vaccinia virus recombinant) were incubated with effector cells for 5 h. E/T ratios were 20:1 for A3, A19, A23, C2, and C2-16 and 15:1 for A26.

recognized. The CD4$^+$ T-cell clones A19, A26, and C2 lysed target cells infected with vP555, vP658, and vP829 (Table 5). This result suggests that the CTL clones A19, A26, and C2 recognize the JEV E protein.

HLA class II restriction of JEV-specific CD4$^+$ T-cell clones. In order to determine the HLA restriction of the JEV-specific CD4$^+$ T-cell clones, we used PBMC from donors A, B, and C as antigen-presenting cells (APCs) in T-cell proliferation assays. Clone A3 proliferated when donor B PBMC and autologous PBMC were used as APCs (Table 6). Thus, we conclude that A3 is restricted by HLA-DQ3, because donors A and B shared only HLA-DQ3. Clones A19, A26, and C2-16 proliferated when PBMC from donors A and C were used as APCs (Table 6). This result suggests that clones A19, A26, and C2-16 are restricted by either HLA-DQ4 or HLA-DR4. The HLA restriction of clone A23 could not be determined, because it proliferated only when stimulated with autologous APCs (Table 6). These results are consistent with those from CTL assays (Table 7). CTL clones A19, A26, C2, and C2-16 lysed vP829-infected donor A and donor C BLCL.

The proliferative responses of clones A19, A23, A26, and C2-16 to JEV Ag were inhibited by anti-HLA-DR antibody but not by anti-HLA-DQ antibody or control antibody (Table 8). These results, along with those shown in Tables 6 and 7, indicate that clones A19, A26, and C2-16 are HLA-DR4 restricted.

TCR V-gene usage by T-cell clones and amino acid sequences of CDR3. We analyzed TCR V-gene usage by the CD4$^+$ T-cell clones. These T-cell clones expressed different, single TCR β chains, but clones A19, A23, and A26 expressed two TCR α chains (Table 9). The amino acid sequences of CDR3 of the TCR α and β chains were also determined. A unique motif was not present on the TCR of the JEV-specific CD4$^+$ T-cell clones.

TABLE 3. Proliferative responses of JEV-specific CD4$^+$ T-cell clones to flavivirus Ag[a]

Clone	[^{3}H]thymidine incorporation, in cpm (SI), after stimulation with the following Ag:							
	JEV	WNV	D1V	D2V	D3V	D4V	Control	None
A3	6,130 (8.0)	1,645 (2.2)	522 (0.7)	647 (0.9)	1,112 (1.5)	707 (0.9)	759	752
A19	1,275 (8.9)	308 (2.1)	105 (0.7)	46 (0.3)	349 (2.4)	386 (2.6)	144	218
A23	2,345 (10.0)	62 (0.3)	164 (0.7)	139 (0.6)	81 (0.4)	122 (0.5)	225	65
A26	3,834 (6.9)	7,933 (14.0)	682 (1.2)	822 (1.4)	547 (1.0)	924 (1.6)	556	918
C2	14,473 (4.1)	3,886 (1.0)	3,141 (0.9)	2,695 (0.8)	3,360 (1.0)	3,640 (1.0)	3,503	3,626
C2-16	11,842 (6.3)	1,490 (0.8)	3,118 (1.6)	1,879 (1.0)	2,430 (1.3)	2,781 (1.5)	1,869	2,500

[a] T-cell clones (1×10^4 to 2×10^4 cells) were cultured with 1:300-diluted flavivirus Ag in the presence of mitomycin C-treated autologous APCs (1×10^5 to 2×10^5 cells) for 3 days. SIs were calculated with the following formula: mean counts per minute of cultures after stimulation with viral Ag/mean counts per minute of cultures after stimulation with control Ag. The data shown are the averages for three wells. The SI is considered to be positive when it is greater than 2.0 and [^{3}H]thymidine incorporation is greater than 1,000 cpm.

TABLE 5. Recognition of JEV E protein by JEV-specific $CD4^+$ CTL clones[a]

Virus with which target cells were infected (JEV genes carried)	% Specific ^{51}Cr release by:		
	A19	A26	C2
vP555 (prM, E, and NS1)	58	36	75
vP658 (E and NS1)	42	27	52
vP829 (prM and E)	47	40	71
vP410 (none [control])	13	0	2

[a] Autologous target cells (10^3) infected with vaccinia virus-JEV recombinants were incubated with effector cells for 5 h. The E/T ratio was 20:1.

DISCUSSION

In the present study, we analyzed JEV-specific $CD4^+$ human T cells induced by immunization with JEV vaccine. Levels of JEV-specific, neutralizing, and hemagglutination inhibition antibodies were increased in the plasma of donors A and C after immunization (data not shown). PBMC were also obtained from donors A and C before vaccination and examined for JEV-specific proliferation. These PBMC did not proliferate after stimulation with JEV Ag, although donors A and C had received the first JEV vaccination a few decades earlier (data not shown). On the other hand, PBMC obtained from the donors 6 to 12 months after the latest JEV vaccination showed significant proliferation after stimulation with JEV Ag.

PBMC from these donors showed cross-reactive proliferative responses to WNV and dengue virus Ag in bulk culture assays. It is unlikely that flavivirus-cross-reactive memory T cells were induced by natural infection with WNV or dengue viruses because the donors had never been to the areas where these viruses were prevalent. Thus, we conclude that immunization with JEV vaccine induces memory T cells which are cross-reactive to WNV and dengue viruses.

We previously reported that primary dengue virus infection induced flavivirus-cross-reactive T lymphocytes as well as dengue virus-specific T lymphocytes (14). We also reported on $CD4^+$ T-cell clones which were cross-reactive for four serotypes of dengue viruses, WNV, and yellow fever virus (14). Flavivirus cross-protection has been reported (7, 8). A high degree of protection was observed for hamsters immunized with JEV and challenged peripherally with WNV (8). JEV-immune animals were fully protected from WNV (7). Cross-reactive $CD4^+$ T cells induced by JEV vaccine in humans may be protective against some other flavivirus infections.

All of the T-cell clones established in the present study recognized E protein. Thus, JEV E protein contains both JEV-specific and JEV and WNV cross-reactive epitopes recognized by human $CD4^+$ T lymphocytes. The homology of E-protein amino acid sequences between JEV and WNV is approximately 80%, while the homology between JEV and dengue viruses is approximately 50%. Although bulk culture proliferation assays suggested the presence of T cells cross-reactive to dengue viruses, the levels of these T cells may have been low because of the low level of amino acid homology. Donors A and C were immunized with inactivated JEV vaccine, which consists of membrane (M), E, and core (C) proteins but has no ability to produce nonstructural proteins. These facts are probably the reasons why all of the T-cell clones recognized E protein. We previously reported that NS3 is the predominant protein recognized by T lymphocytes in dengue virus-infected humans (14). T lymphocytes of donors who are naturally infected with JEV may recognize nonstructural proteins as well as E protein. We are planning to pursue T-cell analysis by using PBMC from donors who are naturally infected with JEV.

We established JEV-specific and flavivirus-cross-reactive $CD4^+$ T-cell clones and analyzed TCR usage by these clones. All of the clones expressed different, single β chains, although some clones expressed two α chains. This result indicates that the $CD4^+$ T-cell clones in the present study originated from different T lymphocytes. Three of the five JEV-specific $CD4^+$ T-cell clones had cytotoxic activity. We previously reported on dengue virus-specific cytotoxic $CD4^+$ T-cell clones (4, 14). The ratio between cytotoxic and noncytotoxic dengue virus-specific $CD4^+$ T-cell clones seemed to vary among donors. It is of interest to know whether there is a difference in the roles of

TABLE 6. Proliferation of $CD4^+$ T-cell clones after stimulation with JEV Ag in the presence of autologous or allogeneic APCs[a]

APC donor	HLA class II type			SI after stimulation of the following clone with JEV Ag:				
	DP	DQ	DR	A3	A19	A23	A26	C2-16
A	4-B1*1401	3,4	4,8	6.8	7.1	9.4	4.1	6.5
B		1,3	2,5	5.2	1.1	1.0	0.7	0.4
C	2-B1*0901	4,6	4,15	1.3	3.1	0.9	4.3	15.0

[a] JEV-specific $CD4^+$ T-cell clones (1×10^4 to 2×10^4 cells) were stimulated with 1:300-diluted JEV Ag in the presence of APCs (1×10^5 to 2×10^5 cells) obtained from donors A, B, and C. SIs were calculated with the following formula: mean counts per minute of cultures after stimulation with JEV Ag/mean counts per minute of cultures after stimulation with control Ag. The data shown are the averages for three wells. [^{3}H]thymidine incorporation levels induced by stimulation with control Ag were 948 cpm with A3, 4,262 cpm with A19, 3,159 cpm with A23, 830 cpm with A26, and 603 cpm with C2-16. The SI is considered to be significant when it is greater than 2.0 and [^{3}H]thymidine incorporation is greater than 1,000 cpm.

TABLE 7. Lysis of vP829-infected allogenic BLCL by $CD4^+$ CTL clones[a]

Target cell donor and infecting virus	% Specific ^{51}C release by:			
	A19	A26	C2	C2-16
A				
vP829	76	59	76	49
vP410	18	0	3	0
C				
vP829	88	62	75	59
vP410	28	5	2	6

[a] E/T ratios were 20:1 for A19, C2, and C2-16 and 15:1 for A26. Assays were done for 5 h.

TABLE 8. Inhibition of proliferation of JEV-specific T-cell clones by anti-HLA-DR antibody[a]

Antibody	Final dilution	[^{3}H]thymidine incorporation (cpm) in:			
		A19	A23	A26	C2-16
Anti-HLA-DQ	1:50	8,769	3,239	3,464	3,524
	1:150	10,851	3,846	3,998	4,383
Anti-HLA-DR	1:50	607	561	378	277
	1:150	907	1,056	711	183
Control immunoglobulin G	1:50	20,598	7,914	8,076	4,667
	1:150	20,258	10,049	10,178	6,823

[a] JEV-specific T-cell clones (1×10^4 to 2×10^4 cells) were cultured with 1:300-diluted JEV Ag or control Ag in the presence of autologous APCs (1×10^5 to 2×10^5 cells). The data shown are the averages for three wells.

TABLE 9. TCR V-gene usage and amino acid sequences of CDR3 of TCR α and β chains

Chain	Clone	V segment	Amino acid sequence in CDR3 region:			J segment
			V	N	J	
α	C2 and C2-16	AV21S	YFCAA	SAE	NYGQNFVFG	AJ26
	A3	AV22S	YFCAL	SDLSS	NTGKLIFG	AJ37
	A19	AV9S1	YCALS	GAS	FNKFYFG	AJ21
		AV20S1	YYCL	VGNIR	GGATNKLIFG	AJ32
	A23	AV2S3	YLCVV	TSAS	DYKLSFG	AJ20
		AV1S3	YFCAVS	PRGP	YNQGGKLIFG	AJ23
	A26	AV6S1	Y	SGGIS	NNLFFG	AJ36
		AV8S1	YFCAAS	TG	GTASKLTFG	AJ44
β	C2 and C2-16	BV9S1	YFCASS	PFLASQGK	GYTFG	BJ1S2
	A3	BV13S5	YFCASS	YQTGC	NQPQHFG	BJ1S5
	A19	BV23S1	YFCASS	PRWGGRD	YEQYFG	BJ2S7
	A23	BV4S1	YLCSVE	TGV	QETQYFG	BJ2S5
	A26	BV18S1	YFCASSP	TPQ	ETQYFG	BJ2S5

cytotoxic and noncytotoxic CD4$^+$ T cells in JEV and dengue virus infections.

The role of flavivirus-cross-reactive CD4$^+$ T cells in secondary flavivirus infections is not completely understood. Neutralizing antibodies play a very important role in protection and recovery from JEV infection. The production of neutralizing antibodies is dependent on CD4$^+$ T cells. It is likely that CD4$^+$ T cells contribute to recovery from flavivirus infections by lysing infected cells and by supporting antibody production. CD4$^+$ T cells may also contribute to the pathogenesis of flavivirus infections. We have reported evidence that dengue virus serotype-cross-reactive CD4$^+$ T lymphocytes may contribute to the pathogenesis of dengue hemorrhagic fever (11). Although there are no data which suggest that flavivirus-cross-reactive CD4$^+$ T lymphocytes also may contribute to the pathogenesis of dengue hemorrhagic fever, epidemiological studies need to be done to answer this question.

A new JEV vaccine should be developed based on the understanding of protective immunity against JEV infection. The vaccine should induce strong protective immunity but should not induce immunity which may lead to immunopathology. It is therefore important to characterize CD4$^+$ T cells induced by a JEV vaccine and by a natural JEV infection and to understand the roles of these T cells in protection and recovery from JEV infections. We are planning to establish more JEV-specific T-cell clones from PBMC of JEV-infected and JEV-vaccinated donors, analyze the functions, and define the epitopes. These studies will provide important information for the development of safer and more efficacious JEV vaccines.

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