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T Cell Responses to an HLA-B*07-Restricted Epitope on the Dengue NS3 Protein Correlate with Disease Severity[1]

Iva Zivna,* Sharone Green,* David W. Vaughn,[2†] Siripen Kalayanarooj,[‡] Henry A. F. Stephens,[§] Dasnayanee Chandanayingyong,[¶] Ananda Nisalak,[†] Francis A. Ennis,* and Alan L. Rothman[3]*

Dengue hemorrhagic fever (DHF), the severe manifestation of dengue virus (DV) infection characterized by plasma leakage, is more common in secondary DV infections in previously infected individuals and is associated with high levels of immune activation. To determine the Ag specificity of this immune response, we studied the response to an HLA-B*07-restricted T cell epitope, residues 221–232 of the DV NS3 protein, in 10 HLA-B*07$^+$ Thai children who were studied during and after acute DV infections. Peptide-specific T cells were detected in 9 of 10 subjects. The frequency of peptide-specific T cells was higher in subjects who had experienced DHF than in those who had experienced DF. We also detected peptide-specific T cells in PBMC obtained at the time of the acute DV infection in 2 of 5 subjects. These data suggest that the NS3 (221–232) epitope is an important target of CD8$^+$ T cells in secondary DV infection and that the activation and expansion of DV-specific T cells is greater in subjects with DHF than in those with dengue fever. These findings support the hypothesis that activation of DV-specific CD8$^+$ T cells plays an important role in the pathogenesis of DHF. ***The Journal of Immunology,*** **2002, 168: 5959–5965.**

Dengue viruses (DV)[4] are a group of mosquito-borne flaviviruses of which there are four distinct serotypes, labeled DV types 1, 2, 3, and 4 (1). The first infection with any of the four serotypes (primary DV infection) is thought to induce life-long immunity to the infecting serotype. However, such individuals are susceptible to infection with other serotypes of DV (secondary DV infection). Primary and secondary DV infections can be asymptomatic or cause illness ranging from dengue fever (DF), a self-limited febrile illness, to dengue hemorrhagic fever (DHF), a severe illness characterized by increased capillary permeability resulting in plasma leakage and hypovolemic shock (2). However, the frequency of DHF is at least 15 times higher, and the frequency of DHF with shock is 80 times higher, in secondary DV infections than in primary DV infections (3–6).

The basis for the increased frequency of DHF during secondary DV infections has not been fully elucidated (7, 8). Several potential mechanisms have been identified. In vitro data indicate that non-neutralizing Abs present before the secondary DV infection can enhance infection of monocytic cells (9). We have noted that primary DV infection also induces T lymphocytes capable of cytolytic function and production of IFN-γ, IL-2, and TNF-α in response to heterologous DV serotypes (8, 10). Plasma levels of IFN-γ and markers of T cell activation, such as soluble TNF receptors, as well as the percentage of circulating CD8$^+$ T cells expressing CD69$^+$, are higher in patients with DHF than in those with DF, suggesting that enhanced activation of these serotype-cross-reactive T cells contributes to DHF pathogenesis (11, 12).

We have previously isolated and characterized a number of human DV-specific CD4$^+$ and CD8$^+$ T cell clones (13–18). T cell epitopes were mapped to multiple DV proteins, with a preponderance of epitopes on the NS3 nonstructural protein. Of interest, DV-specific T cell clones isolated after secondary DV infections were serotype cross-reactive and directed at epitopes on the nonstructural proteins, which show greater sequence homology between serotypes (17). This result is consistent with the hypothesis that memory DV-specific T cells induced by the primary DV infection are preferentially activated during secondary DV infections and may contribute to the pathogenesis of DHF.

Recently developed techniques such as cytokine flow cytometry and HLA-peptide tetramer staining would be invaluable for a more detailed analysis of the activation of DV-specific T cells during secondary DV infection (19, 20). To conduct such analyses requires knowledge of the immunodominant T cell epitopes on DV. We isolated DV-specific T cells from additional subjects who had secondary DV infections and identified T cells specific for the previously defined HLA-B*07-restricted epitope corresponding to amino acids 221–232 of the DV NS3 protein (17). We quantified the T cell response to this peptide in 10 HLA-B*07$^+$ subjects using IFN-γ ELISPOT assays. Peptide-specific T cells were detected in 9 of 10 subjects. The frequency of peptide-specific T cells was higher in subjects who had experienced DHF than in those who had experienced DF. Furthermore, we detected peptide-specific T cells during acute secondary DV infection, consistent

*Center for Infectious Disease and Vaccine Research, University of Massachusetts Medical School, Worcester, MA 01655; †Department of Virology, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand; ‡Queen Sirikit National Institute of Child Health, Bangkok, Thailand; §Institute of Urology and Nephrology, Middlesex Hospital, University College London, London, United Kingdom; and ¶Department of Transfusion Medicine, Siriraj Hospital, Bangkok, Thailand

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[2] Current address: Department of Virus Diseases, Walter Reed Army Institute of Research, Silver Spring, MD 20910.

[3] Address correspondence and reprint requests to Dr. Alan L. Rothman, Center for Infectious Disease and Vaccine Research, Room S5-326, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655. E-mail address: alan.rothman@umassmed.edu

[4] Abbreviations used in this paper: DV, dengue virus; HuABS, human AB serum; BLCL, B lymphoblastoid cell lines; DF, dengue fever; DHF, dengue hemorrhagic fever.

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with the model of accelerated responses of memory DV-specific T cells.

Materials and Methods

Study design and blood samples

Blood samples were obtained from children enrolled in a prospective study of DV infection at Queen Sirikit National Institute of Child Health (formerly Bangkok Children's Hospital; Bangkok, Thailand) and the Kamphaeng Phet Provincial Hospital (Kamphaeng Phet, Thailand) (21). The study population consisted of children, 6 mo–14 years of age, with undifferentiated fever of <72 h duration. The study protocol was approved by the Institutional Review Boards of all participating institutions, and written informed consent was obtained from the parent or guardian of each child.

Acute DV infections were confirmed by both serology and virus isolation from acute phase plasma. Primary and secondary DV infections were distinguished based on the serologic response, following WHO guidelines (22, 23). Clinical diagnoses of DF or DHF were assigned following WHO definitions. Blood samples were obtained during illness; 8 to 11 days after study entry; and at 6 mo, 1 year, 2 years, and 3 years after study entry. PBMC were isolated by density gradient centrifugation, cryopreserved, and stored at −70°C. Frozen PBMC were shipped on dry ice to the University of Massachusetts Medical School (Worcester, MA) for analysis.

For the present study, we identified 10 HLA-B*07$^+$ subjects from among 168 subjects who had confirmed acute DV infections (Table I). Serologic HLA class I typing was performed on fresh blood obtained 6 mo or more after the acute infection at the Department of Transfusion Medicine, Siriraj Hospital (Bangkok, Thailand), using standard techniques (24). The presence of HLA-B*07-related alleles was confirmed by direct and semiautomated sequencing of HLA-B exons 2 and 3 in genomic DNA, using the primers and protocols developed for the 12th International HLA Workshop (25). HLA-B*0705 and *0706 share identical sequences in exons 2 and 3 (which encode the first two external class I protein domains) and thus could not be distinguished, which necessitated using the generic designation of HLA-B*0705/6 for these alleles.

Bulk culture of PBMC

PBMC were thawed, washed, and suspended in 0.5 ml of AIM-V medium (Life Technologies, Gaithersburg, MD) containing 10% heat-inactivated human AB serum (HuABS; Advanced Biotechnologies, Columbia, MD), to which was added 0.5 ml of infectious DV. On day 7, viable cells were recovered and restimulated by incubation with 2×10^6 gamma-irradiated (3700 rad) allogeneic PBMC and anti-CD3 mAb 12F6 (0.1 μg/ml, a gift of Dr. J. Wong) in 0.5 ml of fresh AIM-V medium containing 10% HuABS and 30 U/ml IL-2. Cytolytic activity was assayed after an additional 7–8 days of culture at 37°C.

Isolation of DV-specific T cell clones

DV-specific clones were isolated by limiting dilution as previously described (14, 26). PBMC that had been stimulated with DV in bulk culture for 7 days were collected and plated at concentrations of 1, 3, 10, and 30 cells/well in 96-well round-bottom plates (Costar, Cambridge, MA) in 200 μl of AIM-V medium containing 10% FCS, 10^5 gamma-irradiated allogeneic PBMC, 0.1 μg/ml anti-CD3, and 30 U/ml IL-2. Cells were restimulated every 2 wk. The T cell lines were initially screened for lysis of target cells infected with recombinant vaccinia viruses expressing DV proteins (14, 16). Growing cells that showed DV-specific lytic activity were expanded into 48-well plates. The phenotypes of the cells lines were analyzed using FITC-conjugated anti-CD3, anti-CD4, and anti-CD8 mAb (BD Biosciences, San Diego, CA).

Preparation of target cells

B lymphoblastoid cell lines (BLCLs, $3–5 \times 10^5$ cells) were infected with recombinant vaccinia viruses or wild-type vaccinia virus for 1.5 h at 37°C. The cells were then diluted in 2 ml of RPMI 1640 containing 10% FCS and incubated for a further 12–16 h. Target cells were labeled by incubation with 0.25 mCi $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA) for 60 min at 37°C. After three washes, the target cells were counted and diluted to 1×10^4 cells/ml.

Cytotoxicity assays

Cytotoxicity assays were performed in 96-well round-bottom plates as previously reported (27). Effector cells in 100 μl of RPMI 1640 containing 10% FBS were added to 1000 ^{51}Cr-labeled target cells in 100 μl at E:T ratios of 50:1 to 100:1 for bulk cultures and 5:1 to 20:1 for CTL lines. In CTL assays using synthetic peptides, peptides were added in 50 μl to 10^3 target cells in 100 μl and incubated for 30 min at 37°C, after which 50 μl of effector cells were added. Plates were centrifuged at $200 \times g$ for 5 min and then incubated at 37°C for 4–5 h.

Supernatant fluids were collected with the Skatron supernatant collection system (Molecular Devices, Sunnyvale, CA), and ^{51}Cr content was measured in a gamma counter (Packard Instruments, Downers Grove, IL). Percent specific lysis was calculated as [(experimental ^{51}Cr release − minimum ^{51}Cr release)/(maximum ^{51}Cr release − minimum ^{51}Cr release)] × 100. Assays were performed in triplicate wells. The SEM of samples did not exceed 10%.

ELISPOT assay for IFN-γ-secreting cells

IFN-γ assays were performed as previously described (18, 28) in 96-well filtration plates (Millipore, Bedford, MA) which were coated with mouse anti-human IFN-γ mAb (clone NIB42; BD PharMingen, San Diego, CA). Cryopreserved PBMC were thawed, washed, diluted in RPMI 1640 supplemented with 10% FBS and 2 U/ml recombinant human IL-2, and incubated for 12–15 h. The cells were then washed and added to ELISPOT plates at 3×10^5 per well. The cells were incubated for 16–20 h with or without peptide (10 μg/ml). The plates were then washed and incubated with biotinylated mouse anti-human IFN-γ mAb (clone 4S.B3; BD PharMingen). The ELISPOT plates were developed using fresh substrate buffer (0.3 mg/ml of 3-amino-9-ethylcarbazole and 0.015% H_2O_2 in 0.1 M sodium acetate, pH 5). The precursor frequency of peptide-specific CTL was calculated as the reciprocal of [(number of cells per well)/(mean number of spots in experimental wells − mean number of spots in negative control wells)]. All assays were performed in duplicate. PHA (1/200) was added to positive control wells, and assays were considered valid for analysis only if positive control wells showed >100 spots.

Statistics

Between group comparisons of the frequencies of peptide-specific T cells in PBMC were performed using *t* tests on log-transformed data. Analyses were conducted using SPSS for Windows version 9.0. Values of $p < 0.05$ were considered significant.

Table I. *Characteristics of the study population*

Subject	Diagnosis	Serology	Serotype	HLA Class I Alleles		
				A	B	C
1. C95-029	DF	Primary	D3	24, 34	7 (*0705/6), 75 (*1521)	1, 6
2. C96-089	DF	Secondary	D1	24, 11	7 (*0705/6), 60 (*4011/12)	7
3. C94-115	DF	Secondary	D3	2, 29	7 (*0705/6), 60 (*4011/12)	3
4. C94-062	DF	Secondary	D4	2, 11	7 (*0705/6), 51 (*51011/12)	7, 14
5. K94-015	DF	Secondary	D4	33	7 (*07021), 18 (*1801)	7
6. C94-094	DHF grade 1	Secondary	D4	2, 11	7 (*0705/6), 13 (*1301)	3, 7
7. C94-132	DHF grade 2	Secondary	D4	11, 29	7 (*0705/6), 75 (*1502)	8
8. C95-098	DHF grade 2	Secondary	D1	29	7 (*0705/6), 61 (*4002)	3
9. C94-066	DHF grade 3	Secondary	D1	1, 2	7 (*0705/6), 46 (*4601)	1, 7
10. K94-024	DHF grade 3	Secondary	D2	2, 24	7 (*07021), 46 (*4601)	1, 7

Results

*Isolation of HLA-B*07-restricted CD8$^+$ CTL specific for epitope DV-NS3$_{221–232}$ after acute DV infection from PBMC of subject C94-094*

We previously characterized DV-specific CD8$^+$ CTL from a limited number of individuals who had experienced natural DV infections. To extend these observations, we studied PBMC from subject C94-094, who had experienced acute secondary D4V infection. PBMC stimulated in vitro with DV demonstrated cytolytic activity against autologous BLCL infected with recombinant vaccinia viruses expressing DV NS3 or NS1-2A proteins. This CTL line was cloned by limiting dilution, and wells showing growth were screened for recognition of target cells expressing the D4V NS3 or NS1-2A proteins. Seven NS3-specific CTL clones were identified. All seven CTL clones were CD3$^+$ CD4$^-$CD8$^+$ and recognized target cells expressing the D2V, D3V, and D4V NS3 proteins (data not shown); we did not have a recombinant vaccinia virus available to test for recognition of the D1V NS3 protein.

We defined the HLA restriction of recognition by these CTL clones by testing for lysis of D4V-NS3-expressing allogeneic BLCL that shared one or more HLA class I alleles with autologous BLCL. As shown by representative data in Table II, these CTL clones recognized the DV NS3 protein in the context of HLA-B*07.

We localized the epitope recognized by these HLA-B*07-restricted CTL using recombinant vaccinia viruses expressing portions of the D3V NS3 protein. The CTL clones recognized target cells expressing aa 1–247 of the NS3 protein but did not recognize target cells expressing aa 1–216 (Table III, experiment 1), indicating that the epitope was located approximately between aa 216 and 247 of the NS3 protein. Because we previously identified an HLA-B*07-restricted epitope in this region, we tested for recognition of synthetic peptides corresponding to residues 221–235 of the NS3 protein. The CTL clones recognized this peptide as well as a 12-mer peptide, D4-NS3$_{221–232}$, LAPTRVVAAEME (Table III, experiment 2). Two 9-mer peptides in this region, 221–229 and 222–230, were not recognized by these CTL clones, although the latter peptide contains the motif for peptides binding to HLA-B*07 (29).

*Quantitation of the T cell responses to DV-NS3$_{221–232}$ epitope during and after acute DV infection in a panel of HLA-B*07$^+$ subjects*

DV-specific CTL from different individuals had not previously been shown to recognize the same epitope; therefore, we were interested in determining whether this epitope is an immunodominant T cell target in HLA-B*07$^+$ individuals. We identified 10 HLA-B*07$^+$ subjects with documented acute DV infection from a prospective clinical study of Thai children. We measured the frequency of DV-NS3$_{221–232}$-specific T cells in PBMC obtained 6 mo or more after the acute infection (late convalescence) using IFN-γ ELISPOT assays. In five subjects, we were also able to study PBMC obtained within 10 days of study entry, during or shortly after the febrile period (acute/early convalescent phase). The sequence of this epitope is highly conserved among the four DV serotypes. The sequence is identical in D2V, D3V, and D4V, and there are only two amino acid substitutions, serine for alanine at position 9 of the peptide and alanine for glutamic acid at position 12, in D1V (LAPTRVVASEMA). We used the peptide corresponding to the homologous DV serotypes (Table I) in the assays.

NS3$_{221–232}$-specific T cells were detected in late convalescent PBMC samples from 9 of 10 subjects (Table IV). The frequencies of peptide-specific T cells in these 9 subjects ranged from 1 in 7,000 to 1 in 75,000 PBMC. NS3$_{221–232}$-specific T cells were detected in acute/early convalescent phase PBMC samples from two

Table II. *HLA class I-restricted recognition of dengue NS3 protein by CD8$^+$ CTL lines isolated from subject C94-094*[a]

Target Cell	HLA Class I A	HLA Class I B	HLA Class I C	% Specific Lysis by Indicated T Cell Line 2D6	2D10	1D6
Autologous	2, 11	7	3, 7	**58**	**60**	**63**
TG	23, 29	7, 44	4	**35**	**39**	**41**
GM 3161	3, 3	7, 7		**33**	**35**	**35**
CB	2, 23	35, 44	4	0	0	2

[a] Allogeneic BLCL that shared one or more HLA class I alleles with the autologous cells were infected with vaccinia virus D4:NS3 and used as target cells in ^{51}Cr release assays; shared HLA alleles are underlined. The E:T ratio was 10:1. Boldface values indicate significant target cell lysis.

Table III. *Localization of the epitope on NS3 recognized by CTL isolated from subject C94-094*[a]

Expt.	Target Cells	% Specific Lysis by Indicated T Cell Line 2D6	2D10	2D5	1D6
1	vvD3:NS3/1–618	**48**	**40**	**30**	**51**
	vvD3:NS3/1–354	**63**	**35**	**44**	**54**
	vvD3:NS3/1–247	**61**	**52**	**41**	**63**
	vvD3:NS3/1–216	1	0	1	1
	vvD3:NS3/1–83	0	0	—	—
	vvControl	1	0	0	0
2	D4-NS3$_{221–235}$	ND	**59**	**60**	**77**
	D4-NS3$_{221–232}$	**77**	**49**	**49**	**80**
	D4-NS3$_{221–229}$	0	0	2	0
	D4-NS3$_{222–230}$	0	1	1	1
	D4-NS3$_{224–235}$	1	2	1	0
	No peptide	1	0	2	3

[a] The E:T cell ratios were between 10:1 and 20:1. —, Not tested. In experiment 1, autologous BLCL were infected with recombinant vaccinia viruses (vv) expressing different truncations of the NS3 protein. In experiment 2, autologous BLCL were pulsed with 25 μg of the indicated peptide. Boldface values indicate significant target cell lysis.

Table IV. *T cell responses to the HLA-B*07-restricted epitope dengue-NS3$_{221–232}$*[a]

Group	Subject	Study Day	Peptide-Specific T Cell Frequency
DF	1. C95-029	9	<1/300,000
		365	<1/300,000
	2. C96-089	235	1/27,000
	3. C94-115	9	<1/300,000
		367	1/75,000
	4. C94-062	367	1/13,000
	5. K94-015	722	1/60,000
DHF	6. C94-094	3	1/13,000
		180	1/12,000
		725	1/8,000
	7. C94-132	725	1/7,000
	8. C95-098	9	<1/300,000
		365	1/30,000
	9. C94-066	393	1/7,000
	10. K94-024	9	1/5,000
		1096	1/15,000

[a] The frequency of peptide-specific T cells in PBMC was measured by IFN-γ ELISPOT assay; results are representative of at least two experiments for each subject. Negative controls were PBMC incubated without peptide.

Table V. *Localization of the epitope recognized by NS3-specific T cell lines isolated from subjects C95-098 and C95-029*[a]

Expt.	Target Cells	% Specific Lysis by Indicated T Cell Line				
		C95-098			C95-029	
		7C	9F	11E	1B5	3B5
1	vvD3:NS3/1–618	**51**	**55**	**54**	**29**	**40**
	vvD3:NS3/1–548	**63**	**77**	**68**	**34**	**51**
	vvD3:NS3/447–618	**42**	**57**	**53**	—	—
	vvD3:NS3/1–354	—	—	—	**41**	**48**
	vvD3:NS3/1–205	0	1	3	**38**	**39**
	vvD3:NS3/1–83	—	—	—	**30**	**48**
	vvControl	2	1	0	2	2
2	D2-NS3$_{501–520}$	1	0	2	—	—
	D2-NS3$_{511–530}$	1	0	1	—	—
	D2-NS3$_{521–540}$	**32**	**41**	**45**	—	—
	D2-NS3$_{531–550}$	2	3	3	—	—
3	D2-NS3$_{521–540}$	**43**	**60**	**23**	—	—
	D2-NS3$_{521–537}$	**19**	**17**	**13**	—	—
	D2-NS3$_{521–534}$	1	0	1	—	—
	D2-NS3$_{527–537}$	**42**	**52**	**17**	—	—
	D2-NS3$_{527–536}$	**50**	**68**	**20**	—	—
	D2-NS3$_{527–535}$	**53**	**71**	**22**	—	—
	No peptide	1	2	2	—	—

[a] The E:T ratios were between 10:1 and 20:1. —, Not tested. In experiment 1, autologous BLCL were infected with recombinant vaccinia viruses (vv) expressing different truncations of the NS3 protein. In experiments 2 and 3, autologous BLCL were pulsed with 25 μg of the indicated peptide. Boldface values indicate significant target cell lysis.

of the five subjects studied (Table IV). The frequencies of peptide-specific T cells in acute/early convalescent phase PBMC of these two subjects were approximately equal to or slightly higher than the frequencies of peptide-specific T cells in their late convalescent PBMC. In subjects C94-115 and C95-098, peptide-specific T cells were undetectable in the early convalescent phase PBMC, whereas the frequencies of peptide-specific T cells in their late convalescent PBMC were 1 in 75,000 and 1 in 30,000 PBMC, respectively. Peptide-specific T cells were not detected in either early or late convalescent PBMC from subject C95-029.

*Isolation and characterization of DV-specific CTL clones isolated from PBMC of HLA-B*07$^+$ subjects C95-029 and C95-098*

The above data suggested that the DV-NS3$_{221–232}$ epitope was an immunodominant CTL target in some, but not all, HLA-B*07$^+$ subjects. To evaluate this further, we isolated and characterized DV-specific CTL clones from two of the HLA-B*07$^+$ subjects with low or absent responses to the NS3$_{221–232}$ peptide, using the approach outlined above. We stimulated late convalescent PBMC (obtained 6 mo or more after the acute DV infection) in vitro with both homologous and heterologous DV serotypes, because secondary DV infections involve heterologous DV serotypes.

We isolated 9 DV-specific CD8$^+$ CTL clones from the PBMC of subject C95-029 after stimulation with either D3V (homologous serotype) or D2V (heterologous serotype). All nine CTL clones from this subject recognized target cells expressing the NS3 proteins of D2V, D3V, and D4V (data not shown). We isolated 5 DV-specific CD8$^+$ CTL clones from the PBMC of subject C95-098 after stimulation with D2V (heterologous serotype). All five CTL clones from this subject recognized target cells expressing the NS3 proteins of D2V or D3V, but none recognized target cells expressing the D4V NS3 protein (data not shown). We were not successful in isolating CTL clones from PBMC of this subject after in vitro stimulation with D1V (homologous serotype).

The DV-specific CTL clones isolated from subject C95-029 recognized target cells expressing residues 1–83 of the NS3 protein (Table V). The specific epitope recognized by these CTL clones was not characterized further. The CTL clones also failed to recognize any of the available allogeneic BLCL target cells expressing the DV NS3 protein; therefore, the restricting HLA allele for target cell recognition by these CTL clones could not be defined.

The DV-specific CTL clones isolated from subject C95-098 recognized target cells expressing residues 1–548 or 447–618 of the NS3 protein, demonstrating that the epitope recognized by these CTL clones was located between residues 447 and 548 (Table V). The specific epitope recognized by these CTL clones was defined further using overlapping synthetic peptides that span this region of the NS3 protein. The CTL clones recognized only the peptide corresponding to residues 521–540 (Table V). Using truncations of this peptide, the minimum epitope recognized by these CTL clones was found to correspond to residues 527–535 (GESRKTFVE; Table V). Experiments using allogeneic BLCL pulsed with this peptide demonstrated that target cell recognition by these CTL clones was HLA-B*07-restricted (Table VI). The frequency of T cells specific for this epitope, NS3$_{527–535}$, in PBMC of subject C95-098 was 1 in 41,000 in the early convalescent phase and 1 in 16,000 in the late convalescent phase; we did not have sufficient numbers of PBMC to analyze recognition of this epitope in the other subjects.

Clinical, virologic, and immunogenetic associations with the DV-NS3$_{221–232}$ peptide-specific T cell response

We examined potential factors that might have contributed to the T cell response to the DV-NS3$_{221–232}$ peptide in these subjects. To demonstrate that this peptide could bind to HLA-B*07 from different subjects and stimulate peptide-specific T cells, we determined whether BLCL from these subjects could be recognized by

Table VI. *HLA class I-restricted recognition of dengue NS3 protein by CD8$^+$ CTL lines isolated from subject C95-098*[a]

Target Cells	HLA Class I Alleles			% Specific Lysis by Indicated T Cell Line		
	A	B	C	9F	11E	7C
Autologous	29	7 (*0705/6), 61	3	**68**	**46**	**47**
C95-029	24, 34	7 (*0705/6), 75	1, 6	**26**	**18**	**11**
JK	2, 24	7, 62	3, 7	**27**	**14**	**17**
CLL 040	1, 29	44, 99		1	1	0
HTN#1	2, 11	39, 40	3, 12	0	2	0

[a] Allogeneic BLCL that shared one or more HLA class I alleles with the autologous cells were pulsed with peptide D2-NS3$_{527–535}$ and used as target cells in ^{51}Cr release assays; shared HLA alleles are underlined. Target cells incubated without peptide were not recognized (not shown). The E:T ratio was 10:1. Boldface values indicate significant target cell lysis.

Table VII. *CTL lines isolated from subject C94-094 recognize peptide-pulsed target cells from other HLA-B*07$^+$ subjects*[a]

Target Cells	% Specific Lysis by Indicated T Cell Line	
	2D10	1E7
Autologous	**63**	**48**
C95-029	**58**	**39**
C96-089	**66**	**43**
C94-115	**51**	**30**
C94-132	**54**	**43**
C95-098	**61**	**45**
C94-066	**63**	**47**
No peptide (autologous)	0	1

[a] The E:T ratio was 10:1. Target cells were pulsed with 25 μg of peptide D4-NS3$_{221–232}$ except as noted. Boldface values indicate significant target cell lysis.

a peptide-specific CTL clone. As shown in Table VII, two CTL clones isolated from subject C94-094 could lyse peptide-pulsed BLCL of six other HLA-B*07$^+$ subjects, including subject C95-029, who had no detectable response to this peptide. Furthermore, the peptide-specific T cell responses showed no apparent association with the B*0705/6 or B*07021 alleles (Table I).

We also analyzed potential associations between the NS3$_{221–232}$-specific T cell response and clinical and virologic factors, including the clinical diagnosis, the DV serotype causing infection, and the serologic response (primary vs secondary infection). The frequency of peptide-specific T cells in late convalescent PBMC was higher in subjects who had DHF than in those with DF (Fig. 1). This difference was statistically significant ($p = 0.03$). The frequency of peptide-specific T cells was lower in subjects who had been infected with D3V, but the differences were not statistically significant (Fig. 1). There was a complete concordance between a detectable peptide-specific T cell response and the occurrence of a secondary DV infection; however, there was only one HLA-B*07$^+$ subject with primary DV infection for comparison. When this case was excluded, the difference in the frequency of peptide-specific T cells between subjects with DHF and those with DF remained statistically significant ($p = 0.046$).

Discussion

This is the first quantitative analysis of the T cell response to a specific DV epitope in a cohort of patients with different clinical outcomes of DV infection. Nine of the 10 HLA-B*07$^+$ subjects studied had a detectable response to the NS3$_{221–232}$ peptide. The median epitope-specific T cell frequency in the 9 responders was 1 in 15,000 PBMC. Loke et al. (30) found a comparable frequency of peptide-specific T cells in a small number of subjects following DHF. This is of the same order of magnitude as the responses to T cell epitopes on influenza virus that are considered immunodominant (28, 31); although somewhat lower than the peptide-specific T cell frequencies observed in some persistent virus infections, such as EBV, HIV, CMV, or hepatitis C virus (32–34). Although this 12-mer peptide is longer than peptides directly isolated from HLA-peptide complexes (29) and thus may not represent the optimal HLA-binding peptide, we and others have successfully used longer peptides in IFN-γ ELISPOT assays (35). The high frequency of individuals responding to this DV epitope and the high frequency of peptide-specific T cells in these individuals suggest that this epitope is an important target of the T cell response to DV in naturally infected individuals.

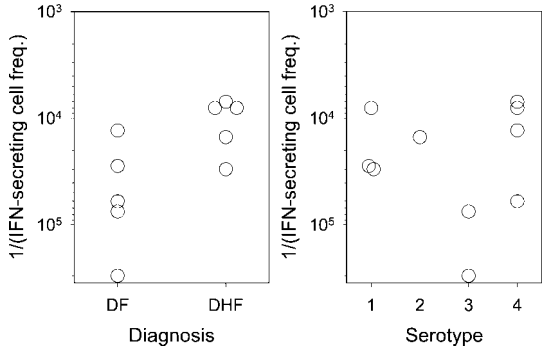

FIGURE 1. Frequency (freq.) of T cells specific for the DV NS3$_{221–232}$ peptide after dengue virus infection in 10 HLA-B*07$^+$ Thai children, by clinical diagnosis and viral serotype. Peptide-specific T cell frequencies were measured by IFN-γ ELISPOT assays using PBMC obtained 6 mo–3 years after the acute DV infection. The frequency of peptide-specific T cells was significantly higher in subjects with DHF than in those with DF ($p = 0.03$ by t test).

We and others previously reported that the nonstructural protein NS3 was a dominant target for flavivirus-specific T cells in humans and mice (13, 14, 16, 18, 30, 36–40). The NS3$_{221–232}$ epitope was also previously reported by our group (17). However, in the previous studies, T cells from different individuals were found to recognize different epitopes. Previous data suggested that both the host HLA allele profiles and the DV serotype causing infection could affect the specificity of DV-specific T cells (41, 42). Our present data showing recognition of the NS3$_{221–232}$ epitope in almost all of the HLA-B*07$^+$ subjects studied, including subjects infected with each of the DV serotypes, suggest that host HLA allele profiles are a more important influence on T cell specificity than DV serotype. This conclusion must be tempered by the recognition that the NS3$_{221–232}$ epitope displays a high degree of sequence homology between DV serotypes, with only two amino acid differences (serine for alanine at residue 229 and alanine for glutamic acid at residue 232) in DV type 1 compared with the other DV serotypes. The T cell response to epitopes with more sequence heterogeneity might be influenced to a greater degree by the DV serotype causing infection. For example, the other HLA-B*07-restricted epitope reported here, NS3$_{527–535}$ is less well conserved: position 3 is alanine in DV types 1 and 2, serine in type 3, and glutamine in type 4; and position 9 is glutamic acid in DV types 1, 3, and 4 but aspartic acid in type 2.

A novel observation from the current data is an association between the magnitude of the peptide-specific T cell response and the severity of clinical disease; the peptide-specific T cell frequency in late convalescent PBMC was significantly higher in the subjects with DHF than in those with DF. This association remained significant after excluding the one subject who was studied after a primary DV infection and who showed no peptide-specific T cell response. Previous studies had demonstrated that DHF was associated with greater activation of CD8$^+$ T cell responses in general (11, 12, 43, 44) but had not shown the association with T cell responses directed at DV epitopes. Because we focused on the response to a single T cell epitope, we cannot say with certainty whether the overall frequency of DV-reactive T cells is higher in subjects with DHF than those with DF. Differences in the frequency of NS3$_{221–232}$ epitope-specific T cells could reflect an increase in the total T cell response to DV or could reflect differences in the dominant epitopes between these two patient groups. In this regard, we noted that DV-reactive T cells could be isolated from the subject with no detectable response to the NS3$_{221–232}$ peptide, and those T cells recognized an epitope located in another part of the NS3 protein. This latter possibility warrants further investigation; if T cell responses to some epitopes are not associated with

DHF, subunit vaccines designed to induce a response to those epitopes could conceivably avoid any increased risk for DHF.

We measured the peptide-specific T cell responses during and after acute DV infections, which were secondary DV infections in 9 of the 10 subjects. Therefore, we cannot determine from the present data whether the higher DV-specific T cell frequency in subjects with DHF indicates that these individuals had a higher pre-existing DV-specific T cell response or whether they had a greater expansion of DV-specific T cells during the acute DV infection. PBMC were not available from before the acute DV infection in these patients. Previously, we found variation from subject to subject in DV-specific T cell responses after primary infection (41), but those subjects were not subsequently challenged with DV and there is no information about subsequent natural exposures to DV in that group. Recently, studies from our group and others have shown that plasma titers of infectious DV or DV RNA are higher in subjects with DHF than in those with DF (45–47). We have also found that immune activation, as measured by plasma levels of soluble TNF receptors, soluble IL-2 receptors, IFN-γ, and IL-10, correlated with peak plasma DV RNA levels and that the peak plasma DV RNA level was the only one of these factors independently associated with the severity of plasma leakage by multiple regression analysis (47). If the levels of DV Ag expression parallel plasma DV RNA levels, it is likely that the higher frequency of DV-specific T cells after DHF is due, at least in part, to greater Ag-driven expansion of the T cell population. At the same time, our preliminary data indicate higher levels of apoptosis in PBMC of subjects with DHF,[5] indicating that T cell expansion during acute DV infection may be balanced by apoptosis, as in experimental models of acute LCMV infection in mice (19, 48).

Structural differences in the HLA-peptide complex did not seem to explain the low or undetectable frequencies of T cells specific for the $NS3_{221–232}$ epitope in some patients. Eight of the 10 subjects shared the HLA B*0705 or B*0706 subtypes, and the frequency of peptide-specific T cells was not significantly different in the 2 subjects with the HLA B*07021 subtype. Further, T cell clones specific for the $NS3_{221–232}$ epitope could recognize peptide-pulsed BLCL from the other HLA-B*07^+ subjects, including those with low or undetectable T cell responses to the peptide (Table VII).

HLA-B*07 and other alleles of the B*07-like "supertype" (HLA-B35, -B51, -B53, -B54, -B55, -B56, -B67, and -B78) are common in Caucasoid, African, and northeast Asian populations (49) but are less well represented in mainland southeast Asians, including the Thais (24). These alleles share a preference for binding peptides characterized by a proline at position 2 and an aromatic or hydrophobic residue at the C terminus and also have similar amino acids forming the B peptide-binding pocket (49). HLA-B*0705/6 and HLA-B*07021 differ by a nonconservative substitution of asparagine (B*0705/6) for aspartic acid (B*07021) at position 114, which is centrally located in the floor of the peptide-binding cleft and contributes to the D-E peptide-binding pocket. However, B*0705/6 and B*07021 have identical amino acids forming the B and F pockets, which could explain the lack of difference in the T cell responses to the NS3 (221–232) epitope between these alleles.

To further analyze the expansion of these DV epitope-specific T cells, we measured the frequency of peptide-specific T cells by IFN-γ ELISPOT in PBMC collected during or early after the acute DV infection in 5 of the 10 subjects. We found it necessary to incubate these PBMC with low concentrations of IL-2 immediately after thawing and before peptide stimulation to detect peptide-specific IFN-γ production; in contrast, the measured peptide-specific T cell frequency in late convalescent PBMC samples was unaffected by the presence or absence of exogenous IL-2. This is consistent with our report that cryopreserved PBMC obtained during or early after acute DV infection were unresponsive to in vitro stimulation and that IL-2 restored their responsiveness to Ag or mitogen stimulation (50). Other groups have found that PBMC collected during acute infections with measles virus or CMV showed reduced in vitro proliferation responses (51, 52). Lechner et al. (34) found that IFN-γ ELISPOT assays significantly underestimated the frequency of hepatitis C virus-specific T cells in PBMC collected during the acute infection; however, they did not study the effect of incubation of PBMC with IL-2.

Subject to the above technical limitation, our data suggest that the expansion of DV epitope-specific T cells occurred early in infection in some subjects (Table IV, subjects C94-094 and K94-024). An accelerated T cell response would likely reflect the activation and expansion of memory DV-specific T cells that were induced by the prior (primary) DV infection. Both of the subjects in whom DV epitope-specific T cells were detected in the acute/early convalescent phase PBMC had DHF, and the third subject with DHF had detectable T cell responses to another DV epitope in the early convalescent phase PBMC. In contrast, neither of the 2 subjects with DF (one with secondary DV infection and one with primary DV infection) had detectable epitope-specific T cells in the early convalescent phase PBMC. The small number of patients studied limits our ability to draw a firm conclusion, however.

Our study was possible because of the availability of a well-characterized cohort of children studied during acute DV infection, with clinical, virologic, and HLA data (21, 23). The data for the first time demonstrate a relationship between the magnitude of the DV-specific T cell response and the severity of acute DV infections. Our study focused on a peptide presented by HLA-B*07. Because HLA-B*07 is relatively uncommon (24), we had a small study cohort. Without further studies, it is uncertain whether the results can be generalized to T cell responses to peptides presented by other, more common HLA alleles (30). Nevertheless, these findings contribute to our understanding of $CD8^+$ T cell responses in relation to the clinical severity of DV infections and have implications for the development of vaccines against DV.

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References

1. Henchal, E. A., and J. R. Putnak. 1990. The dengue viruses. *Clin. Microbiol. Rev. 3:376.*
2. Nimmannitya, S. 1987. Clinical spectrum and management of dengue haemorrhagic fever. *Southeast Asian J. Trop. Med. Pub. Health 18:392.*
3. Halstead, S. B. 1980. Immunological parameters of togavirus disease syndromes. In *The Togaviruses. Biology, Structure, Replication.* R. W. Schlesinger, ed. Academic Press, New York, p. 107.

[5] K. S. Myint, T. P. Endy, D. Mongkolsirichaikul, C. Manomuth, S. Kalayanarooj, D. W. Vaughn, A. Nisalak, S. Green, A. L. Rothman, F. A. Ennis, and D. H. Libraty. Apoptosis of peripheral blood mononuclear cells in children with acute dengue virus infection. *Submitted for publication.*

4. Sangkawibha, N., S. Rojanasuphot, S. Ahandrik, S. Viriyapongse, S. Jatanasen, V. Salitul, B. Phanthumachinda, and S. B. Halstead. 1984. Risk factors for dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak. *Am. J. Epidemiol. 120:653.*
5. Burke, D. S., A. Nisalak, D. E. Johnson, and R. M. Scott. 1988. A prospective study of dengue infections in Bangkok. *Am. J. Trop. Med. Hyg. 38:172.*
6. Thein, S., M. M. Aung, T. N. Shwe, M. Aye, A. Zaw, K. Aye, K. M. Aye, and J. Aaskov. 1997. Risk factors in dengue shock syndrome. *Am. J. Trop. Med. Hyg. 56:566.*
7. Halstead, S. B. 1988. Pathogenesis of dengue: challenges to molecular biology. *Science 239:476.*
8. Rothman, A. L., and F. A. Ennis. 1999. Immunopathogenesis of dengue hemorrhagic fever. *Virology 257:1.*
9. Morens, D. M. 1994. Antibody-dependent enhancement of infection and the pathogenesis of viral disease. *Clin. Infect. Dis. 19:500.*
10. Gagnon, S. J., F. A. Ennis, and A. L. Rothman. 1999. Bystander target cell lysis and cytokine production by dengue virus-specific human $CD4^+$ cytotoxic T lymphocyte clones. *J. Virol. 73:3623.*
11. Green, S., D. W. Vaughn, S. Kalayanarooj, S. Nimmannitya, S. Suntayakorn, A. Nisalak, R. Lew, B. L. Innis, I. Kurane, A. L. Rothman, and F. A. Ennis. 1999. Early immune activation in acute dengue is related to development of plasma leakage and disease severity. *J. Infect. Dis. 179:755.*
12. Green, S., S. Pichyangkul, D. W. Vaughn, S. Kalayanarooj, S. Nimmannitya, A. Nisalak, I. Kurane, A. L. Rothman, and F. A. Ennis. 1999. Early CD69 expression on peripheral blood lymphocytes from children with dengue hemorrhagic fever. *J. Infect. Dis. 180:1429.*
13. Kurane, I., L. C. Dai, P. G. Livingston, E. Reed, and F. A. Ennis. 1993. Definition of an HLA-DPw2-restricted epitope on NS3, recognized by a dengue virus serotype-cross-reactive human $CD4^+CD8^-$ cytotoxic T-cell clone. *J. Virol. 67: 6285.*
14. Livingston, P. G., I. Kurane, L. C. Dai, Y. Okamoto, C. J. Lai, R. Men, S. Karaki, M. Takiguchi, and F. A. Ennis. 1995. Dengue virus-specific, HLA-B35-restricted, human CD8+ cytotoxic T lymphocyte (CTL) clones: recognition of NS3 amino acids 500 to 508 by CTL clones of two different serotype specificities. *J. Immunol. 154:1287.*
15. Gagnon, S. J., W. Zeng, I. Kurane, and F. A. Ennis. 1996. Identification of two epitopes on the dengue 4 virus capsid protein recognized by a serotype-specific and a panel of serotype-cross-reactive human $CD4^+$ cytotoxic T-lymphocyte clones. *J. Virol. 70:141.*
16. Zeng, L., I. Kurane, Y. Okamoto, F. A. Ennis, and M. A. Brinton. 1996. Identification of amino acids involved in recognition by dengue virus NS3-specific, HLA-DR15-restricted cytotoxic $CD4^+$ T-cell clones. *J. Virol. 70:3108.*
17. Mathew, A., I. Kurane, S. Green, H. A. F. Stephens, D. W. Vaughn, S. Kalayanarooj, S. Suntayakorn, D. Chandanayingyong, F. A. Ennis, and A. L. Rothman. 1998. Predominance of HLA-restricted CTL responses to serotype crossreactive epitopes on nonstructural proteins after natural dengue virus infections. *J. Virol. 72:3999.*
18. Zivny, J., M. DeFronzo, W. Jarry, J. Jameson, J. Cruz, F. A. Ennis, and A. L. Rothman. 1999. Partial agonist effect influences the CTL response to a heterologous dengue virus serotype. *J. Immunol. 163:2754.*
19. Murali-Krishna, K., J. D. Altman, M. Suresh, D. J. Sourdive, A. J. Zajac, J. D. Miller, J. Slansky, and R. Ahmed. 1998. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity 8:177.*
20. McMichael, A. J., and C. A. O'Callaghan. 1998. A new look at T cells. *J. Exp. Med. 187:1367.*
21. Kalayanarooj, S., D. W. Vaughn, S. Nimmannitya, S. Green, S. Suntayakorn, N. Kunentrasai, W. Viramitrachai, S. Ratanachu-eke, S. Kiatpolpoj, B. L. Innis, et al. 1997. Early clinical and laboratory indicators of acute dengue illness. *J. Infect. Dis. 176:313.*
22. Anonymous. 1997. *Dengue Haemorrhagic Fever: Diagnosis, Treatment and Control.* World Health Organization, Geneva.
23. Vaughn, D. W., S. Green, S. Kalayanarooj, B. L. Innis, S. Nimmannitya, S. Suntayakorn, A. L. Rothman, F. A. Ennis, and A. Nisalak. 1997. Dengue in the early febrile phase: viremia and antibody responses. *J. Infect. Dis. 176:322.*
24. Chandanayingyong, D., H. A. Stephens, R. Klaythong, M. Sirikong, S. Udee, P. Longta, R. Chantangpol, S. Bejrachandra, and E. Rungruang. 1997. HLA-A, -B, -DRB1, -DQA1, and -DQB1 polymorphism in Thais. *Hum. Immunol. 53:174.*
25. Tilanus, M. G. T., and J. F. Eliaou. 1997. HLA sequencing based typing: strategy and overview. In *Genetic Diversity of HLA: Functional and Medical Implication. Proceedings of the Twelfth International HLA Workshop.*, Vol. 1. D. Charron, ed. EDK Medical and International Publisher, Paris, p. 237.
26. Zivny, J., I. Kurane, A. M. Leporati, M. Ibe, M. Takiguchi, L. L. Zeng, M. A. Brinton, and F. A. Ennis. 1995. A single nine-amino acid peptide induces virus-specific, $CD8^+$ human cytotoxic T lymphocyte clones of heterogenous serotype specificities. *J. Exp. Med. 182:853.*
27. 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.*
28. Jameson, J., J. Cruz, and F. A. Ennis. 1998. Human cytotoxic T-lymphocyte repertoire to influenza A viruses. *J. Virol. 72:8682.*
29. Rammensee, H. G., T. Friede, and S. Stevanovic. 1995. MHC ligands and peptide motifs: first listing. *Immunogenetics 41:178.*
30. Loke, H., D. B. Bethell, C. X. Phuong, M. Dung, J. Schneider, N. J. White, N. P. Day, J. Farrar, and A. V. Hill. 2001. Strong HLA class I-restricted T cell responses in dengue hemorrhagic fever: a double-edged sword? *J. Infect. Dis. 184:1369.*
31. Lalvani, A., R. Brookes, S. Hambleton, W. J. Britton, A. V. Hill, and A. J. McMichael. 1997. Rapid effector function in $CD8^+$ memory T cells. *J. Exp. Med. 186:859.*
32. Callan, M. F., L. Tan, N. Annels, G. S. Ogg, J. D. Wilson, C. A. O'Callaghan, N. Steven, A. J. McMichael, and A. B. Rickinson. 1998. Direct visualization of antigen-specific CD8+ T cells during the primary immune response to Epstein-Barr virus in vivo. *J. Exp. Med. 187:1395.*
33. Barouch, D. H., and N. L. Letvin. 2001. $CD8^+$ cytotoxic T lymphocyte responses to lentiviruses and herpesviruses. *Curr. Opin. Immunol. 13:479.*
34. Lechner, F., D. K. Wong, P. R. Dunbar, R. Chapman, R. T. Chung, P. Dohrenwend, G. Robbins, R. Phillips, P. Klenerman, and B. D. Walker. 2000. Analysis of successful immune responses in persons infected with hepatitis C virus. *J. Exp. Med. 191:1499.*
35. Co, M. D., M. Terajima, J. Cruz, F. A. Ennis, and A. L. Rothman. 2002. Human cytotoxic T lymphocyte responses to live attenuated 17D yellow fever vaccine: identification of HLA-B35-restricted CTL epitopes on nonstructural proteins NS1, NS2b, NS3, and the structural protein E. *Virology 293:151.*
36. 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.*
37. Parrish, C. R., G. Coia, A. Hill, A. Mullbacher, E. G. Westaway, and R. V. Blanden. 1991. Preliminary analysis of murine cytotoxic T cell responses to the proteins of the flavivirus Kunjin using vaccinia virus expression. *J. Gen. Virol. 72:1645.*
38. Hill, A. B., A. Mullbacher, C. Parrish, G. Coia, E. G. Westaway, and R. V. Blanden. 1992. Broad cross-reactivity with marked fine specificity in the cytotoxic T cell response to flaviviruses. *J. Gen. Virol. 73:1115.*
39. Lobigs, M., C. E. Arthur, A. Mullbacher, and R. V. Blanden. 1994. The flavivirus nonstructural protein NS3 is a dominant source of cytotoxic T cell peptide determinants. *Virology 202:195.*
40. Rothman, A. L., I. Kurane, and F. A. Ennis. 1996. Multiple specificities in the murine $CD4^+$ and $CD8^+$ T-cell response to dengue virus. *J. Virol. 70:6540.*
41. Dharakul, T., I. Kurane, N. Bhamarapravati, S. Yoksan, D. W. Vaughn, C. H. Hoke, and F. A. Ennis. 1994. Dengue virus-specific memory T cell responses in human volunteers receiving a live attenuated dengue virus type 2 candidate vaccine. *J. Infect. Dis. 170:27.*
42. Spaulding, A. C., I. Kurane, F. A. Ennis, and A. L. Rothman. 1999. Analysis of murine $CD8^+$ T-cell clones specific for the dengue virus NS3 protein: flavivirus cross-reactivity and influence of infecting serotype. *J. Virol. 73:398.*
43. Kurane, I., B. L. Innis, S. Nimmannitya, A. Nisalak, A. Meager, J. Janus, and F. A. Ennis. 1991. Activation of T lymphocytes in dengue virus infections: high levels of soluble interleukin 2 receptor, soluble CD4, soluble CD8, interleukin 2, and interferon-γ in sera of children with dengue. *J. Clin. Invest. 88:1473.*
44. Green, S., D. W. Vaughn, S. Kalayanarooj, S. Nimmannitya, S. Suntayakorn, A. Nisalak, A. L. Rothman, and F. A. Ennis. 1999. Elevated plasma interleukin-10 levels in acute dengue correlate with disease severity. *J. Med. Virol. 59: 329.*
45. Vaughn, D. W., S. Green, S. Kalayanarooj, B. L. Innis, S. Nimmannitya, S. Suntayakorn, T. P. Endy, B. Raengsakulrach, A. L. Rothman, F. A. Ennis, and A. Nisalak. 2000. Dengue viremia titer, antibody response pattern and virus serotype correlate with disease severity. *J. Infect. Dis. 181:2.*
46. Murgue, B., C. Roche, E. Chungue, and X. Deparis. 2000. Prospective study of the duration and magnitude of viraemia in children hospitalised during the 1996–1997 dengue-2 outbreak in French Polynesia. *J. Med. Virol. 60:432.*
47. Libraty, D. H., T. P. Endy, H. H. Houng, S. Green, S. Kalayanarooj, S. Suntayakorn, D. W. Vaughn, A. Nisalak, A. L. Rothman, and F. A. Ennis. 2002. Differing influences of viral burden and immune activation on disease severity in secondary dengue 3 virus infections. *J. Infect. Dis. In press.*
48. Gallimore, A., A. Glithero, A. Godkin, A. C. Tissot, A. Pluckthun, T. Elliott, H. Hengartner, and R. Zinkernagel. 1998. Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I-peptide complexes. *J. Exp. Med. 187:1383.*
49. Sidney, J., H. M. Grey, R. T. Kubo, and A. Sette. 1996. Practical, biochemical and evolutionary implications of the discovery of HLA class I supermotifs. *Immunol. Today 17:261.*
50. Mathew, A., I. Kurane, S. Green, D. W. Vaughn, S. Kalayanarooj, S. Suntayakorn, F. A. Ennis, and A. L. Rothman. 1999. Impaired T cell proliferation in acute dengue infection. *J. Immunol. 162:5609.*
51. Hirsch, R. L., D. E. Griffin, R. T. Johnson, S. J. Cooper, I. Lindo de Soriano, S. Roedenbeck, and A. Vaisberg. 1984. Cellular immune responses during complicated and uncomplicated measles virus infections of man. *Clin. Immunol. Immunopathol. 31:1.*
52. Carney, W. P., and M. S. Hirsch. 1981. Mechanisms of immunosuppression in cytomegalovirus mononucleosis. II. Virus-monocyte interactions. *J. Infect. Dis. 144:47.*