JCI The Journal of Clinical Investigation

Dominant recognition by human CD8+ cytotoxic T lymphocytes of dengue virus nonstructural proteins NS3 and NS1.2a.

A Mathew, … , M A Brinton, F A Ennis

J Clin Invest. 1996;98(7):1684-1691. https://doi.org/10.1172/JCI118964.

Research Article

A severe complication of dengue virus infection, dengue hemorrhagic fever (DHF), is hypothesized to be immunologically mediated and virus-specific cytotoxic T lymphocytes (CTLs) may trigger DHF. It is also likely that dengue virus-specific CTLs are important for recovery from dengue virus infections. There is little available information on the human CD8+ T cell responses to dengue viruses. Memory CD8+CTL responses were analyzed to determine the diversity of the T cell response to dengue virus and to identify immunodominant proteins using PBMC from eight healthy adult volunteers who had received monovalent, live-attenuated candidate vaccines of the four dengue serotypes. All the donors had specific T cell proliferation to dengue and to other flaviviruses that we tested. CTLs were generated from the stimulated PBMC of all donors, and in the seven donors tested, dengue virus-specific CD8+CTL activity was demonstrated. The nonstructural (NS3 and NS1.2a) and envelope (E) proteins were recognized by CD8+CTLs from six, five, and three donors, respectively. All donors recognized either NS3 or NS1.2a. In one donor who received a dengue 4 vaccine, CTL killing was seen in bulk culture against the premembrane protein (prM). This is the first demonstration of a CTL response against the prM protein. The CTL responses using the PBMC of two donors were serotype specific, whereas all other donors had serotype-cross-reactive responses. For one donor, […]

Dominant Recognition by Human CD8$^+$ Cytotoxic T Lymphocytes of Dengue Virus Nonstructural Proteins NS3 and NS1.2a

Anuja Mathew,* Ichiro Kurane,* Alan L. Rothman,* Ling Ling Zeng,‡ Margo A. Brinton,‡ and Francis A. Ennis*

**Division of Infectious Diseases and Immunology, Department of Medicine, University of Massachusetts Medical Center, Worcester, Massachusetts 01655; and ‡Department of Biology, Georgia State University, Atlanta, Georgia 30303*

Abstract

A severe complication of dengue virus infection, dengue hemorrhagic fever (DHF), is hypothesized to be immunologically mediated and virus-specific cytotoxic T lymphocytes (CTLs) may trigger DHF. It is also likely that dengue virus–specific CTLs are important for recovery from dengue virus infections. There is little available information on the human CD8$^+$ T cell responses to dengue viruses. Memory CD8$^+$CTL responses were analyzed to determine the diversity of the T cell response to dengue virus and to identify immunodominant proteins using PBMC from eight healthy adult volunteers who had received monovalent, live-attenuated candidate vaccines of the four dengue serotypes. All the donors had specific T cell proliferation to dengue and to other flaviviruses that we tested. CTLs were generated from the stimulated PBMC of all donors, and in the seven donors tested, dengue virus–specific CD8$^+$CTL activity was demonstrated. The nonstructural (NS3 and NS1.2a) and envelope (E) proteins were recognized by CD8$^+$CTLs from six, five, and three donors, respectively. All donors recognized either NS3 or NS1.2a. In one donor who received a dengue 4 vaccine, CTL killing was seen in bulk culture against the premembrane protein (prM). This is the first demonstration of a CTL response against the prM protein. The CTL responses using the PBMC of two donors were serotype specific, whereas all other donors had serotype–cross-reactive responses. For one donor, CTLs specific for E, NS1.2a, and NS3 proteins were all HLA-B44 restricted. For three other donors tested, the potential restricting alleles for recognition of NS3 were B38, A24, and/or B62 and B35.These results indicate that the CD8$^+$CTL responses of humans after immunization with one serotype of dengue virus are diverse and directed against a variety of proteins. The NS3 and NS1.2a proteins should be considered when designing subunit vaccines for dengue. (*J. Clin. Invest.* 1996. 98:1684–1691.) Key words: cytotoxic T lymphocyte • dengue virus • flavivirus • epitope analysis

Introduction

Classical dengue, along with its more serious forms, dengue hemorrhagic fever (DHF)[1] and dengue shock syndrome (DSS), are caused by any of the four closely related viruses of the genus *Flaviviridae*, designated dengue virus serotypes 1, 2, 3, and 4 (D1, D2, D3, and D4). Dengue fever is a self-limited febrile disease. DHF is a life-threatening syndrome categorized into four grades from less severe (Gr. 1) to severe (Gr. 4), and is a serious health problem in many parts of the world. DHF grades 3 and 4 are also referred to as DSS because there is profound plasma leakage leading to shock.

Primary infection with any of the four serotypes of dengue virus (DV) induces life-long immunity to that serotype (1), but only short-term immunity to the other dengue serotypes. Epidemiological observations indicate that the more severe form of dengue, DHF, is more likely to occur during secondary dengue infections (2). Serotype–cross-reactive T cells have been shown to be generated after primary dengue infection, supporting the possibility that memory T cells could be activated during secondary dengue infections (3).

Our laboratory has extensively studied the human CD4$^+$ cytotoxic T lymphocyte response to dengue virus infection to attain a deeper understanding of the role of T cells in immunopathology and/or recovery (3–5). The CD8$^+$CTL response, however, has only been extensively examined in one individual who received a live attenuated D4 vaccine (6). CTL clones isolated from this donor were found to recognize a synthetic peptide encompassing amino acids (a.a.) 500–508 of the nonstructural protein NS3 in the context of HLA-B35 (6, 7). CD8$^+$CTLs have been shown to be the principal T cell surveillance system in combating viral infections, and therefore, analysis of the CD8$^+$CTL responses will be critical in elucidating their role in severe dengue infection. The lack of a suitable animal model to analyze severe dengue infection stresses the need to examine the responses of humans. This information should provide useful information for the future development of subunit vaccines.

We have examined the memory CD8$^+$CTL responses in eight healthy adult volunteers who received monovalent (D1, D2, D3, or D4) experimental live attenuated candidate vaccines. PBMC from all donors had significant proliferative responses to flaviviral antigens, and CTLs were generated from the PBMC in all donors. The results indicate that the CD8 T cell response to dengue virus infection is diverse and directed

Address correspondence to Francis A. Ennis, M.D., Division of Infectious Diseases and Immunology, Department of Medicine, University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, MA 01655. Phone: 508-856-4182; FAX: 508-856-4890.

Received for publication 13 May 1996 and accepted in revised form 23 July 1996.

J. Clin. Invest.

0021-9738/96/10/1684/08 $2.00
Volume 98, Number 7, October 1996, 1684–1692

1. *Abbreviations used in this paper:* a.a., amino acid; BLCL, B lymphoblastoid cell line; CTL, cytotoxic T lymphocyte; D1–D4, dengue virus serotypes 1–4, respectively; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; DV, dengue virus; E, envelope protein; NS, nonstructural protein; prM, premembrane protein; WNV, West Nile virus; YFV, yellow fever virus.

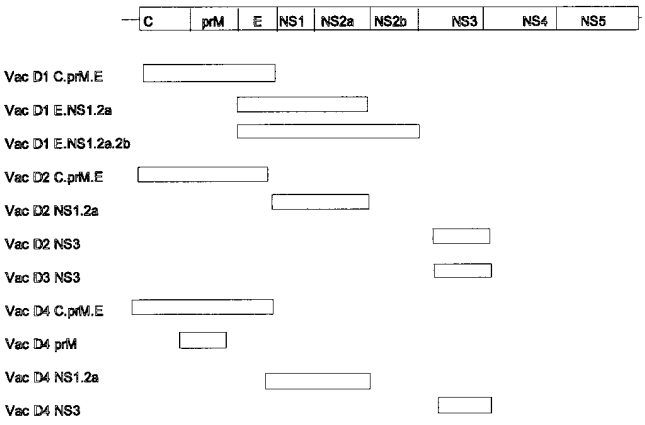

Figure 1. Recombinant vaccinia viruses expressing different portions of the dengue genome. The range of proteins expressed is listed based on the order of proteins in the flavivirus polyprotein, C-prM-E-NS1-NS2a-NS2b-NS3-NS4a-NS4b-NS5, where prM is the precursor of the membrane and is not drawn to scale. The dengue proteins expressed by these recombinant vaccinia viruses are designated based on the dengue serotype that served as the source for cloning and the genome segment included.

against multiple epitopes on various proteins with dominant recognition of NS3 and NS1.2a.

Methods

Viruses. D1 (Hawaii strain) and D2 (New Guinea C strain) were provided by Walter E. Brandt (Walter Reed Army Institute of Research). D3 (CH53489 strain) was provided by Bruce L. Innis (Armed Forces Institute of Medical Science, Bangkok, Thailand). D4 (814669 strain) was provided by Jack McCown (Walter Reed Army Institute of Research, Washington, DC). Yellow fever virus (YFV; 17D strain) was provided by Jacob J. Schlesinger (University of Rochester School of Medicine and Dentistry, Rochester, NY). West Nile virus (WNV; E101 strain) was provided by Margo Brinton (Georgia State University, Atlanta, GA). Viruses were propagated as previously described (8) and frozen at −70°C until use. Recombinant vaccinia viruses containing the gene coding for DV proteins were produced as described previously (9–11). Vaccinia viruses expressing D1 proteins were kindly provided by Dr. Enzo Paoletti (Virogenetics Co., Troy, NY), and those expressing D4 proteins were kindly provided by Dr. C.J. Lai, (National Institutes of Health, Bethesda, MD). D2 and D3 vaccinia recombinants were described earlier (11). Most of the recombinant vaccinia viruses used in this study are shown in Fig. 1. The amino acids contained in the D3 and D4 NS3 truncations are indicated in Tables VI and VII.

Table I. Summary of Vaccination and HLA Class I Types of Immune Donors

Donor number	Serotype and strain of vaccine received	Months after vaccination PBMC obtained	Class I HLA type A	B	Cw
1	D1 45AZ5 (28)	4	2, 11	27, 60	1, 3
2	D2 16881 (17)	12	2, 29	44	5
3	D2 16881 (17)	12	1	8, 38	
4	D2 16881 (17)	12	2, 3	7, 44	5, 7
5	D3 CH5348 (14)	36	2, 24	7, 62	3, 7
6	D4 341750 (13)	22	26, 28	27, 39	1
7	D4 341750 (15)	6	2, 23	35, 44	4
8	D4 341750 (15)	5	2, 28	51, 57	6

Numbers in parentheses are references of the individual vaccines.

Experimental vaccines and PBMC. These donors were proven to be infected with DV by antibody responses and virus isolation. Most of the volunteers had few symptoms (4, 12, 15), and two had symptoms compatible with dengue fever (13, 14, Hoke, C.H., Jr., personal communication). PBMC were purified, resuspended at 10^7/ml in RPMI 1640 with 10% FBS (Sigma Immunochemicals, St. Louis, MO) and 10% DMSO and cryopreserved until use. The HLA typing was done in the Tissue Typing Laboratory at the University of Massachusetts Medical Center in Worcester.

Proliferative responses of PBMC. Proliferation assays were performed as previously described (16). Tritiated thymidine [^{3}H]TdR receptor (TdR) incorporation was counted in a liquid scintillation counter (1205 Betaplate; Pharmacia, Wallac Oy, Finland). Significant virus-specific proliferative responses after immunization were defined using the following criteria (17): the maximum stimulation index (SI) induced by each of the flaviviruses had to be two or more times greater than that induced by control antigen at the same dilution, the differences had to be statistically different, and [^{3}H]thymidine incorporation had to be $>$ 1,000 cpm.

Bulk culture of PBMC. PBMC were cultured at $\sim 5 \times 10^6$/well in 2 ml of AIM-V medium containing 10% human AB serum with the homologous DV at a final dilution of stock virus between 1:2–1:6 in 24-well cluster plates. Cells from donors 2–7 were tested in bulk culture CTL assays between days 7 and 9. Cells from donors 1 and 8 were restimulated with 2×10^6 gamma-irradiated (3,500 rad) autologous PBMC on day 7 in 1 ml of fresh medium containing 10% human AB serum, 10 U/ml IL-2, and DV. Restimulated cells were assayed 7 d later for cytolytic activity.

Preparation of target cells. Lymphoblastoid cell lines (BLCLs) were established by culturing with EBV in 24-well plates (4). BLCLs (5×10^5) were infected with vaccinia viruses for 1.5–2 h at 37°C. The cells were then diluted in 2 ml of media and further incubated for 12–16 h. Target cells were labeled with 0.25 mCi of ^{51}Cr for 60 min at 37°C. After four washes, target cells were counted and diluted to 10^4 cells/ml for use in the cytotoxicity assay. The allogeneic target cells used in the assays were either produced in our laboratory in Massachusetts from unrelated donors, or were obtained from the National Institutes of General Medical Sciences (NIGMS) Human Genetic Mutant Cell Repository or the American Society for Histocompatibility and Immunogenetics (ASHI) Cell Bank and Repository.

^{51}Cr release cytotoxicity assay. Cytotoxicity assays were performed in 96 round-bottom plates, as previously reported (18) . Effector cells were added to 10^3 ^{51}Cr-labeled target cells at an E/T ratio of $\sim$ 100:1. In CTL assays with synthetic peptides, peptides at the indicated concentrations were added to target cells and incubated at 37°C for 30 min, after which the effector cells were added. Plates were centrifuged at 200 *g* for 5 min and incubated for 4–5 h at 37°C. Supernatant fluids were harvested using the supernatant collection system (Skatron Instruments, Sterling, Virginia), and ^{51}Cr content was measured in a gamma counter. The percent-specific ^{51}Cr release was calculated with the following formula: (*cpm experimental release − cpm spontaneous release*)/(*cpm maximum release − cpm spontaneous release*) × 100. All assays were performed in triplicate, and the results were calculated from the average of the triplicate wells. The SEM was $<$ 10% in all the experiments.

mAb depletion assays. Anti-OKT3 (CD3), anti-OKT4 (CD4), anti-OKT8 (CD8) antibodies (Ortho Diagnostic Systems, Inc., Raritan, NJ) and anti-Leu11b antibodies (CD16; Becton Dickinson & Co., Mountain View, CA) were used in antibody-complement depletion assays. DV-stimulated effector cells ($1–1.5 \times 10^6$) were resuspended in 0.5 ml of RPMI 1640 supplemented with 2% FBS with 50 μl of the individual antibodies. After a 30-min incubation at 4°C, the cells were washed twice in cold RPMI/2% FBS and then resuspended in 0.6 ml,

Table II. Proliferation of PBMC from Dengue Vaccine Recipients to Flaviviruses in Bulk Culture

	SI after stimulation with													
	D1V		D2V		D3V		D4V		WNV		YFV		Control‡	
Donors (vaccine)	1:8	1:16	1:8	1:16	1:8	1:16	1:8	1:16	1:8	1:16	1:8	1:16	1:8	1:16
Donor 1 (D1)	5	3	2	1	4	2	1	1	2	2	1	1	1	1
Donor 2 (D2)	26	21	44	55	15	2	15	13	15	14	21	11	5	12
Donor 3 (D2)	7	5	54	40	12	6	12	7	35	37	5	5	15	11
Donor 4 (D2)	5	3	10	14	6	7	5	4	10	4	5	3	2	1
Donor 5 (D3)	3	3	3	2	6	6	2	1	3	3	2	2	3	1
Donor 6 (D4)	1	2	12	11	5	8	19	10	9	5	8	6	4	4
Donor 7 (D4)	1	1	2	2	1	1	2	2	1	2	1	1	1	1
Donor 8 (D4)	3	4	2	3	1	1	10	4	5	3	7	5	4	4

*PBMC (2×10^5 cells) were incubated for 6 d in the presence of serial dilutions of flaviviruses. Cells were pulsed with 1.25 μCi of [^{3}H]TdR for 12 h and [^{3}H] TdR incorporation was measured. SI is calculated as mean cpm of cultures with virus/mean cpm of cultures with medium. Data are maximal SI of immune PBMC of each subject (average of three or two wells). ‡Control, C6/36 mosquito cell supernatants. Underlined values indicate significantly elevated SI values as defined in the Methods section.

to which 0.2 ml of rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) was added. After a 60-min incubation at 37°C, cells were washed three times, resuspended in RPMI/10% FBS, and then used as effector cells in cytotoxic assays.

Results

Lymphocyte proliferative responses to flaviviruses. We first examined the proliferative responses of the PBMC from all eight donors to DV and other flaviviruses. Proliferation was measured on day 7 by uptake of [^{3}H]TdR. Previous studies have shown that both DV-specific $CD4^+$ and $CD8^+$ T cells can be stimulated in this assay (18). All eight donors had significant proliferative responses to one or more of the viruses in the panel (Table II). The PBMC of all the donors responded strongly to the serotype that they had been vaccinated with, and the PBMC of most donors responded to a lesser degree to the other serotypes of DV, WNV or YFV. These results suggest that DV-specific memory T lymphocytes from the eight donors are predominantly serotype-specific, though cross-reactive cells are also present.

Protein specificity of CTLs generated from the PBMC of dengue-immune donors. BLCLs were infected with vaccinia recombinants expressing portions of the D1 genome and used as target cells in the assay (Table III). The effector cells generated from donor 1 PBMC lysed targets expressing the D1 structural proteins core (C), premembrane (prM), and envelope (E), as well as those expressing the E and NS1.2a pro-

*Table III. Recognition of DV Proteins by CTLs Generated from PBMC of D1-immune Donor 1**

	Target cells infected with	Percent specific ^{51}Cr release: Donor 1 (D1)
Exp. 1‡	Vac. D1 (C.prM.E)	58
	Vac. D1 (E.NS1.2a)	51
	Vac. (control)	26
Exp. 2§	Vac. D1 (C.prM.E)	22
	Vac. D1 (E.NS1.2a)	17
	Vac. D2 (E)	4
	Vac. D2 (C.prM)	5
	Vac. D2 (NS1.2a)	7
	Vac. D4 (E)	5
	Vac. (control)	4

*Autologous target cells (10^3) were infected with vaccinia recombinants and incubated with effector cells for 4–5 h. The E/T ratio was ~ 100:1. ‡Donor 1 PBMC were stimulated twice in vitro with D1. §Donor 1 PBMC were stimulated once in vitro with D1.

*Table IV. Recognition of DV Proteins by CTLs Generated from PBMC of D2-Immune Donors 2, 3, and 4 and D3-Immune Donor 5**

	Target cells infected with	Percent specific ^{51}Cr release: Donor 2 (D2)	Donor 3 (D2)	Donor 4 (D2)	Donor 5 (D3)
Exp. 1	Vac.D2 (C. prM.E)	49	35	17	—
	Vac.D2 (C. pr.M)	—	6	10	—
	Vac.D2 (E)	—	25	16	—
	Vac.D2 (NS1.2a)	54	41	92	—
	Vac.D2 (NS3)	71	50	89	—
	Vac.D3 (NS3)	—	—	—	82
	Vac. (control)	26	7	19	32
Exp. 2	Vac.D2 (NS3)	51	43	52	70
	Vac.D3 (NS3)	5	45	17	82
	Vac.D4 (NS3)	16	0	11	54
	Vac (control)	4	0	10	32
Exp. 3‡	Vac.D2 (E)	32	16		
	Vac.D4 (E)	6	0		
	Vac.D2 (NS1.2a)	33	72		
	Vac.D1 (ENS1.2a.2b)		13		
	Vac.D4 (NS1.2a)	11	9		
	Vac. (control)	10	0		

*PBMC from donors 2–5 were stimulated with autologous virus once in vitro. Autologous target cells (10^3) were infected with vaccinia recombinants and incubated with effector cells for 4–5 h. The E/T ratio was ~ 100:1. ‡The E/T ratio was ~ 200:1 for donor 2.

teins (Table III, Exp. 1). PBMC from donors 2–4 were stimulated with D2, and the PBMC from donor 5 was stimulated with D3 for 7–9 d. CTLs from donor 2 recognized target cells expressing the three D2 structural proteins C.prM.E (Table IV, Exp. 1), and were further shown to lyse target cells expressing only E (Table IV, Exp. 3), indicating that specific killing was directed against the E. They also recognized recombinant vaccinia viruses expressing the nonstructural proteins NS1.2a and NS3. CTLs from donor 3 PBMC also recognized E, NS1.2a, and NS3 (Table IV, Exp. 1). CTLs from donor 4 demonstrated no lytic activity against any of the structural proteins, but recognized NS1.2a and NS3 (Table 4, Exp. 1). Using target cells expressing D3NS3, CTLs from donor 5 were found to recognize NS3 (Table IV, Exp. 1).

For donor 6, we found a CTL response against target cells expressing the structural proteins C, prM, and E (Table V, Exp. 1). We then narrowed the response down to the prM protein (precursor of the membrane) using target cells expressing the individual structural proteins. This is the first time a T cell response has been detected against the prM protein. NS3 was also recognized by CTLs from donor 6. CTLs from donor 7 recognized E and NS3 (Table V, Exp. 1). Our laboratory has previously detected $CD8^+$ CTL clones from this donor that recognized NS3 (6, 7). CTLs from donor 8 mainly recognized target cells expressing D4 NS1.2a and E to a lesser degree (Table V, Exp. 1). The results from the above experiments indicate that CTLs were generated against at least one viral protein in all eight donors. The main dengue proteins recognized by CTLs were NS1.2a, NS3, and E.

Serotype specificity of CTLs generated from the PBMC of dengue-immune donors. To analyze the serotype specificity of the CTL responses in all donors, we used target cells expressing dengue proteins of serotypes other than one used to immunize the donor. For donor 1, the response was serotype-specific, since target cells expressing dengue proteins from serotypes other than D1 were not recognized (Table III, Exp. 2). For donor 2, the response to E and NS1.2a proteins was found to be serotype-specific, whereas recognition of NS3 was cross-reactive with D4NS3, but not D3NS3 (Table IV, Exp. 2). CTLs from donor 3 had primarily serotype-specific responses to both E and NS1.2a, but recognition of NS3 was cross-reactive with D3NS3 and not D4NS3 (Table IV, Exp. 2). The response was serotype-specific for CTLs generated against NS1.2a (data not shown) and NS3 (Table IV, Exp. 2) from donor 4 PBMC. CTLs from donor 5 were cross-reactive with both D2 and D4 NS3 (Table IV, Exp. 2).

Using target cells expressing NS3 from serotypes other than D4 for donors 6 and 7, CTLs were shown to be cross-reactive with D3NS3 for donor 6 and with both D2 and D3NS3 for donor 7 (Table V, Exp. 2). CTLs from donor 8 were also cross-reactive as target cells expressing D1E.NS1.2a were lysed (Table V, Exp. 2). These results suggest that the CTLs generated from the donors were predominantly cross-reactive although donors 1 and 4 had serotype-specific responses.

Localization of the epitope within the NS3 protein recognized by CTLs from donors 3, 5, 6, and 7. Donor 3 PBMC recognized D2 and D3NS3 (Table VI, Exp. 2) to similar levels. We used recombinant vaccinia viruses expressing truncated D3NS3 proteins to localize the epitopes on NS3 recognized by this donor's CTLs. The epitope was localized to a region between a.a. 247 and 618, since a truncated vaccinia recombinant containing a.a. 1–247 (Vac D3-8) of D3NS3 was not recognized (Table VI, Exp. 1). From Exp. 2, the area was further localized to the regions from a.a. 247-412, since Vac D3-C2 (a.a. 412–618) was not recognized. Exp. 3 narrows the epitope down to a region between a.a. 247 and 354. Similarly for donor 5, it appears that the epitope lies between a.a. 214 and 247 (Table VI, Exp. 2).

From the results in Table VII Exp. 1, we conclude that

*Table V. Determination of Proteins Recognized by CTLs Generated from PBMC of D4-immune Donors 6–8**

	Target cells infected with	Percent specific ^{51}Cr release: Donor 6 (D4)	Donor 7 (D4)	Donor 8 (D4)
Exp. 1	Vac. D4 (C.prM.E)	29	41	16
	Vac. D4 (prM)	26	27	
	Vac. D4 (E)	11	51	14
	Vac. D4 (NS1.2a)	7	27	32
	Vac. D4 (NS3)	30	58	8
	Vac. (control)	0	21	0
Exp. 2	Vac. D2 (NS3)	−2	59	
	Vac. D3 (NS3)	16	42	
	Vac. D4 (NS3)	24	73	
	Vac. D2 (NS1.2a)			11
	Vac. D1 (E.NS1.2a)			46
	Vac. (control)	1	26	−1

*PBMC were stimulated with D4V twice for donor 6 and once for donors 7 and 8, as described in Methods. E/T ratio was ~ 100:1.

*Table VI. Localization of the Epitopes on NS3 Recognized by CTLs Generated from PBMC of D2-immune Donor 3 and D3-immune Donor 5**

	Target cells infected with	a.a.‡	Percent specific ^{51}Cr release: Donor 3 (D2)	Donor 5 (D3)
Exp. 1	Vac.D2 (NS3)	1–618	32	—
	Vac.D3 (NS3)	1–618	26	—
	Vac.D3-8	1–247	3	—
	Vac.D3-3	1–214	4	—
	Vac.D3-14	1–176	2	—
	Vac. (control)		0	—
Exp. 2	Vac.D3 (NS3)	1–618	46	54
	Vac.D3-5	1–548	51	60
	Vac.D3-3	1–214	—	39
	Vac.D3-C1	447–618	8	42
	Vac.D3-C2	412–618	9	—
	Vac. (control)		7	34
Exp. 3	Vac.D3 (NS3)	1–618	76	78
	Vac.D3-15	1–354	54	87
	Vac.D3-8	1–247	—	71
	Vac. (control)		3	29

*PBMC were stimulated with autologous virus once in vitro and used as effectors. Autologous target cells (10^3) were infected with vaccinia recombinants and incubated with effector cells for 4–5 h. The E/T ratio was ~ 100:1. ‡The numbers indicate a.a. residues of NS3 expressed by the various recombinant vaccinia viruses.

Table VII. Localization of the Epitope on NS3 Recognized by PBMC of D4-immune Donors 6 and 7

	Target cells infected or pulsed with	a.a.*	Percent specific ^{51}Cr release	
			Donor 6 (D4)	Donor 7 (D4)
Exp. 1	Vac.D4.NS3	1–618	23	58
	Vac.D4 (1–452)	1–452	18	29
	Vac. D4 (183–452)	183–452	4	—
	Vac. D4 (453–618)	453–618	5	58
	Vac. (control)		−4	21
Exp. 2	Vac.D4 (453–618)		—	54
	Vac. (control)		—	24
	Pep.D2.500	500–508	—	46
	Pep.D3.500	500–508	—	41
	Pep.D4.500	500–508	—	62
	None		—	−3

*The number indicates amino acid regions on D4 NS3 expressed by the recombinant vaccinia viruses. Peptides were present at a final concentration of 25 μg/ml for the duration of the assay.

CTLs from donor 6 recognized an epitope between a.a. 1 and 183 of NS3. The CTLs of donor 7, on the other hand, recognized a region between a.a. 453 and 618 on NS3. Using synthetic peptides, we confirmed that the recognition was directed against a peptide containing a.a. 500–508 of NS3 (Table VII, Exp. 2). This is in agreement with previous work in our laboratory which showed that $CD8^+$CTL clones isolated from this donor recognized a synthetic peptide containing a.a. 500–508 of NS3 (6). CTLs in bulk culture were also shown to lyse synthetic peptides containing a.a. 500–508 of D2 and D3NS3, confirming the cross-reactive nature of this response at the peptide level (Table VII, Exp. 2).

Phenotype of dengue-specific CTLs in bulk cultures. To identify the phenotypes of DV-specific cytotoxic T cells in each of the donors, cell depletion studies with mAbs and complement were carried out. The assays were done 7–9 d after stimulation with autologous virus for donors 2–5, 7, and 8. For donors 1 and 6, effector cells were restimulated on day 7 and used on day 14. The effector cell populations were tested against target cells infected with vaccinia recombinants (expressing portions of the dengue genome that had elicited significant lysis in previous experiments), a vaccinia control, and a natural killer cell-sensitive tumor cell line, K562. For donor 2, autologous BLCLs persistently infected with D2 were used as targets.

*Table VIII. Characterization of Cytotoxic T Cells Generated in Bulk Cultures**

Donor	Target cells infected with	Treatments					Phenotype of cells
		C′	Anti–CD3 + C′	Anti–CD4 + C′	Anti–CD8 + C′	Anti–CD16 + C′	
Donor 1 (D1)	Vac.D1 (E.NS1.2a)	33	2	0	21	16	CD4
	Vac.D1 (C.prM.E)	36	6	9	15	29	CD4 + CD8
	Vac. (control)	1					
	K562	29	24	12	30	12	
Donor 2 (D2)	D2V infected	18	0	19	0	21	CD8
	Uninfected	−1					
	K562	19	9	12	14	7	
Donor 3 (D2)	Vac. D2 (NS1.2a)	34	3	27	2	38	CD8
	Vac. D2 (NS3)	20	−1	15	3	18	CD8
	Vac. (control)	0					
	K562	10	5	8	10	2	
Donor 5 (D3)	Vac. D3 (NS3)	21	8	14	9	14	CD8
	Vac. (control)	10					
	K562	36	21	24	24	7	
Donor 6 (D4)	Vac. D4 (nonM)	26	0	15	−1	17	CD8
	Vac. (control)	0					
	K562	13	4	7	11	1	
Donor 7 (D4)	Vac.D4$(NS3)_{453\text{-}613}$	52	9	31	−4	23	CD8
	Vac. D4 (E)	22	5	10	−1	6	CD8, CD4
	Vac. (control)	0					
	K562	11	11	7	5	0	
Donor 8 (D4)	Vac. D4 (NS1.2a)	27	0	33	5	26	CD8
	Vac. (control)	5					
	K562	12	4	12	6	0	

*Values are the percent specific ^{51}Cr release from target cells. Underlined values are those that were decreased by > 50% compared to controls with the indicated treatment. PBMC were stimulated with autologous virus for 7 to 14 d as described in Methods. The E/T ratio was between 100 and 150:1 before treatments.

Treatment with anti-CD3 antibody and complement depleted the specific killing activity (Table VIII). These results indicate that the killing was mediated by T cells in all donors. For donor 1, the killing of target cells expressing the envelope and nonstructural proteins NS1.2a (infected with recombinant Vac D1E.NS1.2a) was mediated by $CD4^+$CTLs, because depletion with anti-CD4 and complement decreased the killing substantially. Killing of donor 1 targets infected with a construct containing all three structural proteins (Vac D1C.prM.E; Table VIII) was mediated by $CD4^+$ and $CD8^+$CTLs, since depletions with either anti-CD4 or anti-CD8 decreased killing. For donors 2, 3, and 5–8, killing was mediated almost exclusively by $CD8^+$CTLs, since treatment with anti-CD8 and complement significantly decreased the lytic activity. No other antibody had a significant effect on the levels of lysis for these donors. Taken together, the results suggest that stimulation of immune PBMC in vitro with infectious DV activated mainly $CD8^+$ memory CTLs, and the levels of $CD8^+$CTL activity varied between donors.

*Table IX. Determination of HLA Class I Restriction in Recognition of Dengue Proteins by $CD8^+$ CTLs**

	Target	HLA A	Class I B	Type CW	Percent specific‡ cytotoxicity	Potential restricting allele
Donor 2						
(Vac D2E)	Autologous	2,29	44	5	18	
	JK	2,24	7,62	3,7	9	
	CB	2,3	35,44	4	24	B44
	TomG	23,29	7,44	4	25	
	9038	2	18	7	−8	
Donor 2						
(Vac D2NS1.2a)	Autologous	2,29	44	5	28	
	JK	2,24	7,62	3,7	9	
	CB	2,23	35,44	4	14	B44
	VA17	2,25	18,44	5	19	
	9038	2	18	7	0	
Donor 2						
(Vac D2NS3)	Autologous	2,29	44	5	71	
	CB	2,23	35,44	4	73	B44
	9038	2	18	7	−11	
	9049	33	65	8	5	
Donor 3						
(Vac D3NS3)	Autologous	1	8,38		73	
	VA 12	1,24	35	4	10	B38
	GM3105A	26,28	18,38		49	
	GM3162	2	8,35		12	
Donor 5						
(Vac D3NS3)	Autologous	2,24	7,62	3,7	24	
	UM27	24,30	13,62	3,6	16	
	63390	3,24	7,62	4,7	17	
	Puzzro	2,3	39	2,3	3	A24 and/or
	Autologous	2,24	7,62	3,7	52	B62
	CB	2,23	35,44	4	12	
	CP	2,28	51,57	6	1	
	9022	1	8	7	16	
	VA16	2,28	7,14	5	9	
Donor 7						
(Pulsed with	Autologous	2,23	35,44	4	52	
a.a. 500–508	VA03	2,24	7,35	3,7	32	B35
of D4NS3)	JC	3,24	35	4	45	

*PBMC from donor 2 were stimulated with D2V for 7 d and used as effectors. (10^3) allogeneic targets were infected with the indicated vaccinia recombinants with effectors, and were incubated for 4–5 h. Shared HLA antigens are underlined. ‡For clarity, values are given as the percent of specific cytotoxicity for the vaccinia recombinant–infected targets—percent of specific cytoxicity for vaccinia control–infected targets.

HLA restriction of the lysis of target cells by $CD8^+$ bulk culture CTLs. HLA restriction of the $CD8^+$ bulk cultures was examined in four immune donors to identify the predominant restricting alleles in these cultures. Target cells were infected with the recombinant vaccinia viruses that had previously elicited significant lysis and a control vaccinia virus. From Table IX, we conclude that recognition of E, NS1.2a, and NS3 by the PBMC of donor 2 were all restricted by HLA-B44, since only allogeneic target cells having HLA-B44 in common with the autologous BLCLs showed significant lysis.

The nonstructural protein NS3 was recognized by CTLs of a majority of our donors; therefore, we tested the HLA restriction of three additional donors that had NS3-specific CTLs. The results indicate that recognition of NS3 by donor 3 CTL was B38 restricted. For donor 5, it appears that both A24 and/or B62 may restrict recognition of D3NS3. In bulk culture, $CD8^+$CTLs for donor 7 lysed allogeneic targets pulsed with a.a. 500–508 that have only HLA B35 in common with autologous targets, confirming what we observed earlier with the $CD8^+$CTL clones of this donor.

Discussion

In this study, we analyzed the bulk culture $CD8^+$CTL responses of eight donors who received monovalent, live-attenuated candidate dengue vaccines (D1, D2, D3, or D4). Our aim was to analyze the diversity of the $CD8^+$T cell responses in dengue infection. The PBMC from all donors exhibited proliferative responses to flaviviruses, with the most significant proliferation being to the serotype of virus contained in the vaccine that they had received and a variable level of cross-reactive proliferation to other dengue serotypes or to other flaviviruses. We detected dengue-specific CTL activity in all eight donors, indicating that these live-attenuated vaccines elicited powerful dengue-specific CTL responses. We found CTL killing against a wide variety of proteins, including E, prM, and NS1.2a and NS3 of DV, as summarized in Table X. Seven of eight donors had $CD8^+$CTL responses directed against one or more of these proteins although the levels of lysis varied between donors.

In DV-immunized inbred mice, bulk culture $CD8^+$CTLs recognized only a very limited number of epitopes on DV proteins—as few as one to three on the entire genome that encodes 3,386 amino acids (19). Preliminary studies indicated that there were a limited number of $CD8^+$CTL epitopes on the dengue protein in humans as well (6). The results reported in this paper illustrate that there are multiple $CD8^+$CTL epitopes on both structural and nonstructural proteins, which is similar to our previous results demonstrating that multiple epitopes

Table X. Summary of CTLs Generated in Cultures from PBMC of Dengue-immune Donors 1–8

Donor	Serotype of vaccine received	Proliferation	Dengue proteins recognized by CTL	Serotype specificity or cross-reactivity	Localization of epitope	Responding cell type (mAb+C^1)	Potential HLA-restricting allele
1	D1	+	C.prM.E	Specific	—	CD4+CD8	ND
			E.NS1.2a	Specific	—	CD4	ND
2	D2	+	E	Specific	—		B44
			NS1.2a	Specific	—	CD8 (virus-	B44
			NS3	Cross-reactive	—	infected targets)	B44
3	D2	+	E	Specific	—	ND	ND
			NS1.2a	Specific	—	CD8	ND
			NS3	Cross-reactive	247–354	CD8	B38
4	D2	+	NS1.2a	Specific	—	ND	ND
			NS3	Specific	ND	ND	ND
5	D3	+	NS3	Cross-reactive	214–247	CD8	A24 and/or B62
6	D4	+	prM	Specific	—	CD8	ND
			NS3	Cross-reactive	1–183	ND	—
7	D4	+	E	Specific	—	CD8+CD4	ND
			NS3	Cross-reactive	500–508	CD8	B35
8	D4	+	NS1.2a	Cross-reactive		CD8	ND

were recognized by DV specific $CD4^+$CTLs in both humans and mice (3–5, 20).

The demonstration that DV-specific memory CTLs are serotype cross-reactive after a primary dengue infection supports the possibility that these memory T cells can be activated in secondary infections with a heterologous serotype of virus (21). We have previously shown that T cell activation markers, soluble IL-2 receptor, soluble CD4, and soluble CD8 are elevated in the sera of patients with DHF compared to those with DF, implying that these activated T cells are likely to play a role in severe disease (22). Lymphokines released from these activated cells are likely to have immunoregulatory roles.

We have previously isolated human $CD4^+$CTL clones from different donors with varying patterns of virus specificities (3, 11, 13, 20) and $CD8^+$CTL clones from one donor with four patterns of serotype specificity (7). The results from this current study on eight vaccine donors indicate that human $CD8^+$ bulk cultures have multiple specificities. We detected virus-specific CTLs from two of eight donors (donors 1 and 4, Table X), as well as CTLs with various subcomplex specificities that recognized D2 and D4 NS3 (donor 2); D2 and D3NS3 (donor 3); D2, D3, and D4 (donors 5 and 7); and D3 and D4NS3 (donor 6). Donor 8 generated CTLs that were cross-reactive primarily with D1ENS1.2a and, to a much lesser degree, with D2NS1.2a. Data from these experiments further lends support to the possibility that both $CD4^+$ and $CD8^+$CTLs are capable of being activated in secondary infections with a different serotype of virus, and may contribute to the pathogenesis seen in DHF.

All donors had CTLs that recognized at least one nonstructural protein (NS3 or NS1.2a). The predominant protein that was recognized by the PBMC of the eight donors was NS3. NS3 is the second largest viral protein (containing 618 amino acids) and is one of the most highly conserved proteins among flaviviruses. It has both a protease (NH_2 terminus) and a nucleotide triphosphatase/helicase (COOH terminus) activity. Our laboratory has previously identified multiple sites on NS3 recognized by human $CD4^+$CTLs (3,11). In the murine system, we have shown that NS3 is the major target for DV-specific H-2^k CTL (19). Here, we show that there are at least five sites on NS3 (different from those recognized by $CD4^+$CTLs) recognized by $CD8^+$CTLs. NS3 is therefore an immunodominant protein, and although there is a predominance of recognition of determinants on this protein, there is no single immunodominant epitope. The results from Table X indicate that CTLs recognizing NS3 are usually cross-reactive, whereas CTLs recognizing other proteins (E, prM, and NS1.2a) are mainly serotype specific. The cross-reactive nature of CTLs against NS3 may be caused by the high levels of conservation of amino acids of NS3 among the different serotypes. Since NS3 is recognized by a wide variety of immune donor CTLs, and antibodies to NS3 are unable to mediate antibody-dependent enhancement of infection (23), this protein is an attractive candidate for a subunit vaccine. It is also possible that NS3-specific CTL may contribute to the immunopathology of DHF, since they can be reactivated after exposure to a heterologous serotype of virus. We do not yet have vaccinia recombinants that express the nonstructural proteins NS4a, NS4b, and NS5; therefore, there may be CTLs against these proteins that we have not detected.

Genetically mediated factors may also play a critical role in accounting for the differences in susceptibility to severe infection. Since NS3 was the immunodominant protein recognized by CTLs, the MHC restriction was examined using CTLs generated in bulk cultures of four donors to identify class I alleles that might present different portions of the NS3 genome. For donors 2, 3, 5, and 7, the potential restricting alleles for NS3 are B44, B38, A24 and/or B62, and B35.

It is interesting that for donor 2, $CD8^+$ T cell epitopes for all three proteins (E, NS3, and NS1.2a) were recognized in the context of HLA-B44. A peptide-binding motif has been identi-

fied for HLA-B44 with an acidic amino acid (glutamic acid) at P2 and a less rigid requirement for a basic or hydrophobic amino acid at P9 (24). It is likely that if we were to identify nonamer peptides with the above motif in the three proteins, one or more of them would be able to sensitize target cells for lysis. Using the HLA-B44 motif, three different antigenic peptides from both influenza NS1 and NP proteins were shown to be presented by HLA-B44 to antiinfluenza type A–specific CTLs from one donor (24). There are numerous factors including proteolytic mechanisms, peptide stability, transport, selective binding, etc., which determine the selection of particular peptides presented on one type of class I allele (25, 26). In one study done on Thai children who had been hospitalized with severe dengue infection, HLA A2 and HLA B17 appeared to be statistically associated with developing increased risk of severe disease (27).

There are a wide variety of factors that may contribute towards the development of DHF and DSS. Vaccine strategists therefore need to carefully weigh the options, since they have to develop a vaccine that stimulates protective responses without increasing the risk of immunopathological consequences. Considerable work still needs to be done to establish the roles of CD4 and CD8 T cells in viral clearance and pathology. These studies will guide scientists in the development of a protective and effective vaccine that may prevent DV infections.

Acknowledgments

We thank Kim Wojnowski for assistance in typing the manuscript. We also thank Jurand Janus for preparation of viral antigens.

This work was supported by grants NIH RO1-AI30624, NIHT32-A1 07272, and NIH PO1-AI34533 from the National Institutes of Health and V22/181/76 from the World Health Organization. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

References

1. Halstead, S.B. 1988. Pathogenesis of dengue. Challenges to molecular biology. *Science (Wash. DC).* 239:476–481.

2. Halstead, S.B. 1988. Immunological parameters of togavirus disease syndromes. *In* The Togaviruses: Biology, Structure, Replication. R.W. Schlesinger, editor. Academic Press, New York. 107–173.

3. Kurane, I., M.A. Brinton, A.L. Samson, and F.A. Ennis. 1991. Dengue virus-specific, human $CD4^+$ $CD8^-$ cytotoxic T-cell clones: multiple patterns of virus cross-reactivity recognized by NS3-specific T-cell clones. *J. Virol.* 65:1823–1828.

4. Green, S., I. Kurane, R. Edelman, C.O. Tacket, K.H. Eckles, D.W. Vaughn, C.H. Hoke, and F.A. Ennis. 1993. Dengue virus-specific human $CD4^+$ T-lymphocyte responses in a recipient of an experimental live-attenuated dengue virus type 1 vaccine: bulk culture proliferation, clonal analysis and precursor frequency determination. *J. Virol.* 67:5962–5967.

5. Zivny, J., I. Kurane, C.O. Tacket, R. Edelman, and F.A. Ennis. 1993. Dengue virus-specific human $CD4^+$ cytotoxic T lymphocytes generated in short term culture. *Viral Immunol.* 6:143–151.

6. 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 B-35 restricted, human $CD8^+$ cytotoxic T lymphocyte (CTL) clones: recognition of NS3 amino acids 500–508 by CTL clones of two different specificities. *J. Immunol.* 154:1287–1295.

7. Zivny, J., I. Kurane, A. 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.* 182:853–863.

8. Kurane, I., D. Hebblewaite, W.E. Brandt, and F.A. Ennis. 1984. Lysis of dengue virus infected cells by natural cell mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity. *J. Virol.* 52:223–230.

9. Falgout, B., R. Chanock, and C.J. Lai. 1989. Proper processing of dengue virus nonstructural protein NS1 requires N terminal hydrophobic signal sequence and the downstream nonstructural protein NS2a. *J. Virol.* 63:1852–1860.

10. Zhao, B., G. Prince, R. Horswood, K. Eckels, R.M. Chanock, and C.J. Lai. 1989. Expression of dengue virus structural proteins and nonstructural protein NS1 by a recombinant vaccinia virus. *J. Virol.* 64:4109.

11. Zeng, L.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: 3108–3117.

12. Bhamarapravati, N., S. Yoksan, T. Chayaniyayothin, S. Angsubphakorn, and A. Bunyaratvej. 1987. Immunization with a live attenuated dengue 2 virus candidate vaccine (16681-PDK 53): clinical, immunological and biological responses in adult volunteers. *Bull. WHO.* 65:189–195.

13. 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 crossreactive human $CD4^+$ cytotoxic T-lymphocyte clones. *J. Virol.* 70:141–147.

14. Innis, B.L., K.H. Eckels, E. Kraiselbard, D.R. Dubois, G.F. Meadors, D.J. Gubler, D.S. Burke, and W.H. Bancroft. 1988. Virulence of a live dengue virus vaccine candidate: a possible new marker of dengue virus attenuation. *J. Infect. Dis.* 158:876–880.

15. Hoke, C.J., Jr., F.H. Malinozki, K.H. Eckels, S.R. McNair, D.R. Dubois, P.L. Summers, T. Simms, J. Burrows, S.E. Hasty, and W.H. Bancroft. 1990. Preparation of attenuated dengue 4 (341750 Carib.) virus vaccine. II. Safety and immunogenicity in humans. *Am. J. Trop. Med. Hyg.* 43:219–226.

16. Kurane, I., B.L. Innis, A. Nislak, C. Hoke, S. Nimmannitya, A. Meager, and F.A. Ennis. 1989. Human T cell responses to dengue virus antigens: proliferative responses and production. *J. Clin. Invest.* 83:506–513.

17. Dharakul, T., I. Kurane, N. Bhamarapravati, S. Yoksan, D. Vaughn, C.H. Hoke, and F.A. Ennis. 1994. Dengue virus specific memory T cell responses in human volunteers receiving a live attentuated dengue virus type 2 candidate vaccine. *J. Infect. Dis.* 170:27–33.

18. Bukowski, J.F., I. Kurane, C.J. Lai, M. Bray, B. Falgout, and F.A. Ennis. 1989. Dengue virus-specific cross reactive $CD8^+$ human cytotoxic T lymphocytes. *J. Virol.* 63:5086–5091.

19. Rothman, A.L., I. Kurane, C.J. Lai, M. Bray, B. Falgout, R. Men, and F.A. Ennis. 1993. Dengue virus protein recognition by virus-specific murine CD8+ cytotoxic T lymphocytes. *J. Virol.* 67:801–806.

20. Kurane, I., A. Meager, and F.A. Ennis. 1989. Dengue virus specific human T cell clones: serotype cross-reactive proliferation, interferon production, and cytotoxic activity. *J. Exp. Med.* 170:763–775.

21. Kurane, I., and F.A. Ennis. 1992. Immunity and immunopathology in dengue virus infections. *Semin. Immunol.* 4:121–127.

22. 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: 1473–1480.

23. Kurane, I., and B.J. Mady. 1991. Antibody dependent enhancement of dengue virus infection. *Rev. Med. Virol.* 1:211–221.

24. DiBrino, M., K. Parker, D. Margulies, J. Shiloach, R. Turner, W. Biddison, and J. Coligan. 1995. Identification of the peptide binding motif for HLA-B44, one of the most common HLA-B alleles in the caucasian population. *Biochemistry.* 34:10130–10138.

25. Barber, L.D., and P. Parham. 1994. The essence of epitopes. *J. Exp. Med.* 180:1191–1194.

26. Elliot, T., M. Smith, P. Driscoll, and A. McMichael. 1993. Peptide selection by class I molecules of the major histocompatibility complex. *Curr. Biol.* 3: 854–866.

27. Chiewslip, P., R. Scott, and N. Bhamarapravati. 1981. Histocompatibility antigens and dengue hemorrhagic fever. *Am. J. Trop. Med. Hyg.* 30:1101–1105.

28. McKee, K.T., Jr., W.H. Bancroft, K.H. Eckels, R.R. Redfield, P.L. Summers, and P.K. Russel. 1987. Lack of attenuation of a candidate dengue 1 vaccine (45AZ5) in human volunteers. *Am. J. Trop. Med. Hyg.* 36:435–442.