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Definition of an HLA-DPw2-Restricted Epitope on NS3, Recognized by a Dengue Virus Serotype-Cross-Reactive Human CD4$^+$ CD8$^-$ Cytotoxic T-Cell Clone

ICHIRO KURANE,[1]* LI-CHEN DAI,[1] PETER G. LIVINGSTON,[1] ELAINE REED,[2] AND FRANCIS A. ENNIS[1]

Division of Infectious Diseases and Immunology, Department of Medicine, University of Massachusetts Medical Center, Worcester, Massachusetts 01655,[1] and Department of Pathology, College of Physicians and Surgeons of Columbia University, New York, New York 10032[2]

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We previously reported that the clone JK34 was cross-reactive for dengue virus types 1, 2, 3, and 4 and recognized NS3 (I. Kurane, M. A. Brinton, A. L. Samson, and F. A. Ennis, J. Virol. 65:1823–1828, 1991). In the present experiments, we defined the epitope at the amino acid level, with 93 15-mer overlapping peptides which cover the entire NS3. A peptide 4 which contains amino acids 251 to 265 of NS3 sensitized the autologous B lymphoblastoid cell line (LCL) to the lysis by JK34. The smallest peptide recognized by JK34 was a 10-mer peptide which contains amino acids 255 to 264 (EIVDLMCHAT). A monoclonal antibody to HLA-DP inhibited the lysis of epitope peptide-pulsed autologous LCL by JK34. Genotypic typing revealed that the HLA-DP of this donor is DPA1*01, DPB1*0201, which is serologically defined as HLA-DPw2. JK34 lysed peptide 4-pulsed allogeneic LCL which carried HLA-DPw2. These results indicate that HLA-DPw2 is the restriction allele for recognition of this epitope by JK34.

Dengue virus infections are a serious cause of morbidity and mortality in many areas of the world: southeast and south Asia, Central and South America, and the Caribbean (9, 11). Dengue virus infection can be asymptomatic or cause two forms of disease (8). Dengue fever is a self-limited febrile disease. In some situations, patients infected with dengue virus leak plasma into interstitial spaces, resulting in hypovolemia and sometimes circulatory collapse. This severe life-threatening syndrome is termed dengue hemorrhagic fever (DHF).

Development of dengue virus vaccines is a potential method for preventing dengue virus infections; however, the epidemiological observations have shown that DHF is observed much more commonly in secondary dengue virus infections than in primary infections (1, 9). These observations raise the possibility that dengue virus vaccine may induce immune responses which could lead to the immunopathology of DHF. Dengue virus vaccines should induce protective immune responses but should not induce immunity which may increase the risk of DHF during future dengue virus infections. Although protective immune mechanisms against dengue viruses are not understood, it is believed that serotype-specific neutralizing antibodies can prevent dengue virus infections and that dengue virus-specific cytotoxic T lymphocytes (CTL) contribute to recovery from infection. Therefore, experimental subunit vaccines should include neutralizing B-cell epitopes and dominant CTL and helper T-cell epitopes.

We have reported that the NS3 protein was recognized by the majority of CD4$^+$ CTL clones established from a dengue virus-infected individual (14). Identification and characterization at the amino acid levels of these CD4$^+$ CTL epitopes will provide useful information for the future development of dengue vaccines. In this paper, we define an epitope on NS3 recognized by a dengue virus serotype-cross-reactive CD4$^+$ CD8$^-$ CTL clone, JK34, with overlapping synthetic peptides.

Dengue virus type 1, Hawaii strain; type 2, New Guinea C strain; type 3, CH53489 strain; and type 4, 814669 strain; yellow fever virus (17D strain); and West Nile virus (E101 strain) were used. Dengue virus, yellow fever virus, and West Nile virus antigens were prepared by using dengue virus-infected Vero cells as previously reported (16). Synthetic peptides of the NS3 protein of dengue virus type 4, 814669 strain (17), were synthesized with the RaMPS system (DuPont, Boston, Mass.) as previously reported (6, 23). The peptides consist of 15 amino acids (aa) which overlapped each other by 7 to 10 residues.

Cytotoxic assays were done as previously reported (14). In cytotoxic assays using synthetic peptides, 10^3 cells in 0.1 ml were incubated with peptide in 0.05 ml for 30 min, and effector cells in 0.05 ml were then added to each well. After incubation at 37°C for 6 h, the supernatant fluid was collected from each well and counted in an automatic gamma counter. The percent specific ^{51}Cr release was calculated by the following formula: 100 × (counts per minute of experimental release − counts per minute of spontaneous release)/(counts per minute of maximal release − counts per minute of spontaneous release). Concentrations of the peptide which induce 50% maximum lysis were calculated on the basis of the dose-response curves with peptide at concentrations from 25 μM to 2.5 × 10^{-6} μM, and the percent specific lysis of dengue virus type 3 antigen-cultured target cells was considered as the maximum lysis in the experiment. The 10th International Histocompatibility Workshop lymphoblastoid cell lines (LCL) (10w9023, -9011, -9029, -9038, -9022, -9052, and -9077) (American Society for Histocompatibility and Immunogenetics, Lenexa, Kans.) were used in HLA-DP restriction experiments.

The HLA-DPA1 and -DPB1 genotype of the donor was established by polymerase chain reaction amplification of

* Corresponding author.

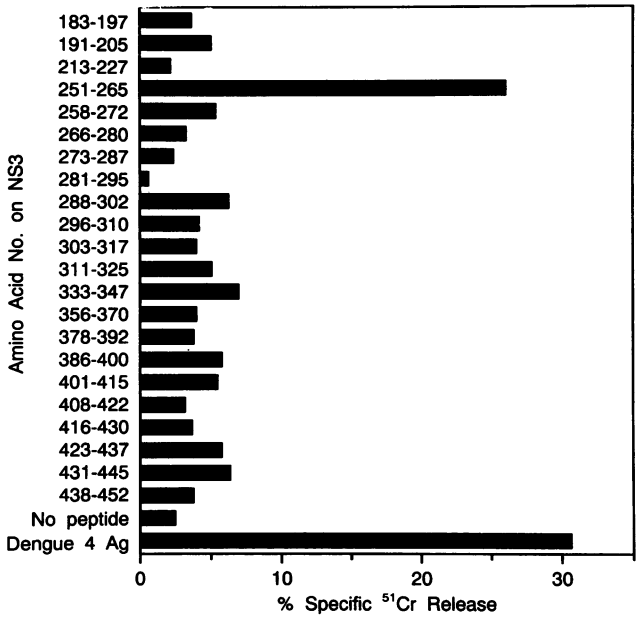

FIG. 1. Recognition of a peptide 4 (aa 251 to 265) by a dengue serotype-cross-reactive CD4+ CTL clone, JK34. A total of 10^3 autologous LCL were incubated with 8×10^3 JK34 cells (effector/target ratio = 8:1) for 6 h in the presence of peptides at 20 μM. Dengue 4 Ag, dengue virus type 4 antigen.

the first domain of DPA1 and DPB1 genes and hybridization with sequence-specific oligonucleotide probes as previously described (19, 20).

The establishment and partial characterization of the JK34 clone have been already reported (14). Briefly, JK34 was established from the peripheral blood mononuclear cells of a donor who had been infected with dengue type 3 virus (CH53489) 1 year earlier, with a limiting dilution technique. JK34 has a $CD3^+$ $CD4^+$ $CD8^-$ phenotype and has dengue virus-specific cytotoxic activity. JK34 is cross-reactive for dengue virus types 1, 2, 3, and 4 but not for yellow fever virus or West Nile virus.

TABLE 2. HLA-DP-restricted lysis of the target cells by JK34[a]

Monoclonal antibody added[b]	% Specific ^{51}Cr release		
	Dengue virus type 3 antigen	Peptide 4	None
None	76	62	0
Anti-HLA-DP	2	8	ND[c]
Anti-HLA-DQ	83	57	ND
Anti-HLA-DR	90	52	ND
Anti-HLA class I	87	66	ND

[a] A total of 2.5×10^3 autologous LCL were incubated with 2.5×10^4 JK34 cells (effector/target ratio = 10:1) for 6 h in the presence of monoclonal antibodies at a final dilution of 1:80. The peptide at 25 μM was included in the cytotoxic assay.

[b] B7/21, S3/4, OKIa1 (Ortho Diagnostic Systems, Inc., Raritan, N.J.), and W6/32 (Accurate Biochemical Co., Westbury, N.J.) were used as anti-HLA-DP, anti-HLA-DQ, anti-HLA-DR, and anti-HLA class I, respectively.

[c] ND, not determined.

After demonstrating that clone JK34 recognized the NS3 protein (14), we attempted to determine the epitope recognized by JK34, with 93 15-mer overlapping peptides which covered the entire NS3 protein. Only peptide 4 which contains aa residues 251 to 265 (HTGREIVDLMCHATF) sensitized autologous LCL to the lysis by JK34 (Fig. 1). None of the other peptides sensitized autologous LCL to the lysis by JK34 (data not shown). To further delineate the smallest peptide recognized by this clone, we synthesized N- and C-terminal truncations of peptide 4 as shown in Table 1. Peptides 4a, 4b, 4c, 4d, 4f, and 4k sensitized autologous LCL to the lysis by JK34. These results indicate that the smallest peptide recognized by JK34 is located on aa 255 to 264, which have the amino acid sequence of EIVDLMCHAT.

HLA restriction in the recognition of the epitope by JK34 was first examined with monoclonal antibodies to HLA molecules (Table 2). A monoclonal antibody to HLA-DP inhibited the lysis of peptide 4-pulsed target cells and dengue virus type 3 antigen-cultured target cells by JK34, but monoclonal antibodies to HLA-DQ, HLA-DR, and HLA class I did not. This result confirmed that recognition of the epitope by JK34 is HLA-DP restricted as previously reported (14). In order to determine HLA-DP allelic restric-

TABLE 1. Determination of the core epitope recognized by JK34 with truncated synthetic peptides

Peptide	Amino acids on NS3	Amino acid sequence	% Specific ^{51}Cr release[a]	Peptide concn (μM) for 50% maximum lysis[b]
4	251–265	HTGREIVDLMCHATF	50	2.5
4a	252–265	TGREIVDLMCHATF	44	4.4
4b	253–265	GREIVDLMCHATF	40	8.3
4c	254–265	REIVDLMCHATF	50	3.5
4d	255–265	EIVDLMCHATF	34	11
4e	256–265	IVDLMCHATF	11	>25
4f	251–264	HTGREIVDLMCHAT	26	23
4g	251–263	HTGREIVDLMCHA	9	>25
4h	251–262	HTGREIVDLMCH	2	>25
4i	251–261	HTGREIVDLMC	1	>25
4j	251–260	HTGREIVDLM	0	>25
4k	255–264	EIVDLMCHAT	28	17
4l	255–263	EIVDLMCHA	0	>25
4m	256–264	IVDLMCHAT	3	>25
None			0	

[a] Percent specific ^{51}Cr release at the peptide concentration of 25 μM. Effector/target ratio was 14:1. The assay was for 6 h.

[b] Peptide concentrations which induce 50% maximum lysis were calculated on the basis of dose-response curves as stated in the text. Percent specific lysis of dengue virus type 3 antigen-cultured autologous LCL was 51%.

TABLE 3. HLA-DPw2-restricted lysis of peptide 4-pulsed allogeneic LCL by JK34[a]

Target	HLA-DP	Genotype		% Specific ^{51}Cr release[b]
		DPA1	DPB1	
Autologous	w2	01	0201	89
9023	w1	02	0101	4
9011	w2, w4	01	0201, 0401	40
9029	w2	01	0201	20
9038	w2	01	0201	40
9022	w3	01	0301	4
9052	w4	01	0401	5
9077	w5	NA[c]	NA	0

[a] A total of 10^3 autologous or allogeneic LCL were incubated with 1.2×10^4 JK34 cells (effector/target ratio = 12:1) for 6 h in the presence of peptide 4 at 25 μM.
[b] Percent specific ^{51}Cr release of autologous LCL without peptide 4 was 0.
[c] NA denotes data not available.

tion, the HLA-DP alleles of this donor were defined by oligonucleotide typing. The results revealed that this donor is homozygous for DPA1*01, DPB1*0201, which is serologically defined as HLA-DPw2. HLA-DP-typed allogeneic LCL were then used in CTL assays. Allogeneic LCL which express HLA-DPw2 and were pulsed with peptide 4 were lysed by JK34, but allogeneic LCL which express other HLA-DP alleles were not (Table 3). These results indicate that recognition of this epitope by JK34 is restricted by HLA-DPw2.

The NS3 genes of dengue virus types 1, 2, 3, and 4 have been sequenced (7, 13, 17, 18). The epitope determined in this study is completely conserved among dengue virus types 1, 2, 3, and 4, which is compatible with the dengue serotype cross-reactivity of this clone. This clone did not recognize yellow fever virus or West Nile virus. Yellow fever virus has three substitutions, I to V at aa 256, V to I at aa 257, and L to A at aa 259 (21), and West Nile virus has one substitution, L to V at aa 259 (2, 24).

The epitopes recognized by HLA-DP-restricted, human $CD4^+$ T-cell clones have only been defined for a few viruses. HLA-DPw4-restricted epitopes on hepatitis B virus, rabies virus, and human immunodeficiency virus type 1 were identified (3, 4, 10). The result reported in the present paper is, however, the first definition of the HLA-DPw2-restricted epitope recognized by human $CD4^+$ T cells. The core of the HLA-DPw4-restricted epitopes on hepatitis B virus and human immunodeficiency virus type 1 has been reported to be 10 and 12 aa residues, respectively (3, 10), whereas the smallest peptide recognized by HLA-DPw2-restricted T-cell clone JK34 contains 10 aa residues. There is a stretch of 5 aa residues which is conserved among those three HLA-DPw4-restricted epitopes, and this area of homology contains a motif of Arg-X-Leu, where X is a hydrophobic amino acid (10). The HLA-DPw2-restricted epitope on dengue virus has no homology with those HLA-DPw4-restricted epitopes.

Some naturally processed peptides bound to major histocompatibility complex class II molecules have been isolated from human and murine cells and been sequenced (5, 12, 22). Chicz et al. have reported that peptides isolated from HLA-DR1 were 13 to 25 aa long and that these peptides displayed a high degree of heterogeneity both in length and the site of terminal truncation (5). Rudensky et al. showed that peptides isolated from $I\text{-}A^b$ and $I\text{-}E^b$ were 13 to 17 aa long and discussed not finding simple patterns of amino acids in peptides (22). Hunt et al. reported that peptides isolated from $I\text{-}A^d$ were 16 to 18 aa long and contained a 6-residue binding motif (12). Although we have determined that the clone recognizes a peptide that is 10 aa long, the naturally processed peptide which is recognized by JK34 would be longer. Isolation of the naturally processed epitope from dengue virus-infected autologous cells will be important to perform in the future.

The role of dengue virus-specific $CD4^+$ CTL in dengue virus infections is poorly understood. It is likely that $CD4^+$ CTL contribute to prevention of infection and recovery from dengue virus infection by helping the generation of neutralizing antibodies and $CD8^+$ CTL and by lysing dengue virus-infected HLA class II-bearing cells. It is also possible that these $CD4^+$ CTL contribute to the pathogenesis of DHF by lymphokine production and cytotoxic activities (15). If $CD4^+$ CTL do contribute to prevention and recovery, the epitopes recognized by these dengue serotype-cross-reactive clones should be included in the subunit vaccines. It is therefore important to map the epitopes recognized by other dengue virus-specific T-cell clones and determine the HLA restriction of these clones. This information will be useful in the development of safe and effective dengue virus vaccines in the future.

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REFERENCES

1. **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**:172–180.
2. **Castle, E., T. Nowak, U. Leidner, G. Wengler, and G. Wengler.** 1985. Sequence analysis of the viral core protein and the membrane-associated protein V1 and NV2 of the flavivirus West Nile virus and the genome sequence for these proteins. Virology **145**:227–236.
3. **Celis, E., and R. W. Karr.** 1989. Presentation of an immunodominant T-cell epitope of hepatitis B surface antigen by the HLA-DPw4 molecule. J. Virol. **63**:747–752.
4. **Celis, E., J. Larson, L. Otvos, Jr., and W. H. Wunner.** 1990. Identification of a rabies virus T cell epitope on the basis of its similarity with a hepatitis B surface antigen peptide presented to a T cell by the same MHC molecule (HLA-DPw4). J. Immunol. **145**:305–310.
5. **Chicz, R. M., R. G. Urban, W. S. Lane, J. C. Gorga, L. J. Stern, D. A. A. Vignali, and J. L. Strominger.** 1992. Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogeneous in size. Nature (London) **358**:764–768.
6. **Dai, L. C., K. West, R. Littaua, K. Takahashi, and F. A. Ennis.** 1992. Mutation of human immunodeficiency virus type 1 at amino acid 585 on gp41 results in loss of killing by $CD8^+$ A24-restricted cytotoxic T lymphocytes. J. Virol. **66**:3151–3154.
7. **Fu, J., B.-H. Tan, E.-H. Yap, Y.-C. Chan, and Y. H. Tan.** 1992. Full-length cDNA sequence of dengue type 1 virus (Singapore strain S275/90). Virology **188**:953–958.
8. **Halstead, S. B.** 1980. Immunological parameters of togavirus disease syndromes, p. 107–173. *In* R. W. Schlesinger (ed.), The togaviruses: biology, structure, replication. Academic Press, Inc., New York.
9. **Halstead, S. B.** 1988. Pathogenesis of dengue: challenges to molecular biology. Science **239**:476–481.
10. **Hammond, S. A., E. Obah, P. Stanhope, C. R. Monell, M. Strand, F. M. Robbins, W. B. Bias, R. W. Karr, S. Koenig, and R. F. Siliciano.** 1991. Characterization of a conserved T cell epitope in HIV-1 gp41 recognized by vaccine-induced human

cytotoxic T cells. J. Immunol. **146:**1470–1477.
11. **Hayes, E. B., and D. J. Gubler.** 1992. Dengue and dengue hemorrhagic fever. Pediatr. Infect. Dis. J. **11:**311–317.
12. **Hunt, D. F., H. Michel, T. A. Dickinson, J. Shabanowitz, A. L. Cox, K. Sakaguchi, E. Appella, H. M. Grey, and A. Sette.** 1992. Peptides presented to the immune system by the murine class II major histocompatibility complex molecule I-A^d. Science **256:** 1817–1820.
13. **Irie, K., P. M. Mohan, Y. Sasaguri, R. Putnak, and R. Padmanabhan.** 1989. Sequence analysis of cloned dengue virus type 2 genome (New Guinea-C strain). Gene **75:**197–211.
14. **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.
15. **Kurane, I., and F. A. Ennis.** 1992. Immunity and immunopathology in dengue virus infections. Semin. Immunol. **4:**121–127.
16. **Kurane, I., B. L. Innis, A. Nisalak, C. Hoke, S. Nimmanitya, A. Meager, and F. A. Ennis.** 1989. Human T cell responses to dengue virus antigens. Proliferative responses and interferon gamma production. J. Clin. Invest. **83:**506–513.
17. **Mackow, E., Y. Makino, B. Zhao, Y.-M. Zhang, L. Markoff, A. Bucker-White, M. Guiler, R. Chanock, and C.-J. Lai.** 1987. The nucleotide sequence of dengue type 4 virus: analysis of genes coding for nonstructural proteins. Virology **159:**217–228.
18. **Osatomi, K., and H. Sumiyoshi.** 1990. Complete nucleotide sequence of dengue type 3 virus genome RNA. Virology **176:** 643–647.
19. **Reed, E., E. Ho, F. Lupu, P. McManus, R. Vasilescu, A. Foca-Rodi, and N. Suciu-Foca.** 1992. Polymorphism of HLA in the Romanian population. Tissue Antigens **39:**8–13.
20. **Reed, E., F. Lupu, P. McManus, R. Seigle, and N. Suciu-Foca.** 1992. Population and family studies of HLA-DR4 by use of oligonucleotide typing. Tissue Antigens **39:**266–271.
21. **Rice, C. M., E. M. Lenches, S. R. Eddy, S. J. Shin, R. L. Sheets, and J. H. Strauss.** 1985. Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. Science **229:**726–733.
22. **Rudensky, A. Y., P. Preston-Hurlburt, S.-C. Hong, A. Barlow, and C. A. Janeway, Jr.** 1991. Sequence analysis of peptides bound to MHC class II molecules. Nature (London) **353:**622–627.
23. **Takahashi, K., L.-C. Dai, T. R. Fuerst, W. E. Biddison, P. L. Earl, B. Moss, and F. A. Ennis.** 1991. Specific lysis of human immunodeficiency virus type 1-infected cells by a HLA-A3.1-restricted $CD8^+$ cytotoxic T lymphocyte clone that recognize a conserved peptide sequence within the gp41 subunit of the envelope protein. Proc. Natl. Acad. Sci. USA **88:**10277–10281.
24. **Wengler, G., E. Castle, U. Leidner, T. Nowak, and G. Wengler.** 1985. Sequence analysis of the membrane protein V3 of the flavivirus West Nile virus and of its gene. Virology **147:**267–274.