# 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

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Yellow fever virus (YFV) is a reemerging problem despite the existence of an effective live-attenuated vaccine. The induction of YFV-neutralizing antibodies undoubtedly contributes to vaccine efficacy, but T lymphocyte responses to YFV likely play a role in long-term efficacy. We studied the T lymphocyte responses to YFV in four vaccinees. Proliferation and cytolytic responses to YFV were demonstrated in all subjects. We isolated 13 YFV-specific CD8$^+$ CTL lines that recognized epitopes on the E, NS1, NS2b, and NS3 proteins; eight CTL lines were HLA-B35-restricted. YFV-specific T cell responses were detectable by IFN$\gamma$ ELISPOT assays 14 days postvaccination, with T cell frequencies sustained for up to 19 months. To our knowledge, this is the first report of human T lymphocyte responses following YFV vaccination. These results indicate that the live 17D YFV vaccine induced CD8$^+$ T cell responses directed against at least four different HLA-B35-restricted YFV epitopes.   © 2002 Elsevier Science (USA)

*Key Words:* yellow fever virus; vaccine; HLA-B35; flavivirus; cytotoxic T lymphocyte; epitope; E protein; NS1 protein; NS2b protein; NS3 protein.

## INTRODUCTION

Yellow fever virus (YFV) is a member of the family Flaviviridae, which includes at least 68 single-stranded RNA viruses transmitted by mosquitoes or ticks. Other members of this family include dengue, Japanese encephalitis, West Nile, and hepatitis C viruses. Yellow fever is once again emerging as an important infectious disease in Africa, South America, and Central America. According to the WHO, the 18,735 cases with 4522 deaths reported in the endemic areas of Central and South America during the period of 1987–1991 were the highest since 1948 (Robertson *et al.,* 1996). YFV causes a clinical illness that can be divided into three stages. Following an incubation period of 3–6 days, patients experience high fevers accompanied by prostration, headache, and malaise lasting 3–4 days. After a 24- to 48-h period during which symptoms remit, 15% of patients enter the third stage of illness (period of intoxication) marked by jaundice, fever, relative bradycardia, and a hemorrhagic diathesis, which can progress to multiorgan failure involving the liver, kidney, and heart (Monath, 1999).

Although antivirals such as interferon and ribavirin as well as certain amaryllidaceae compounds have shown promise *in vitro* (Gabrielsen *et al.,* 1992; Monath, 1987), treatment for yellow fever remains supportive. Public health efforts have concentrated on preventive measures that focus on the administration of the YFV vaccine to persons who may be exposed to YFV through work, travel, or habitation in endemic areas.

The YFV vaccine, a live attenuated vaccine (strain 17D) derived from the human isolate Asibi, has been administered to over 300 million people with a minority of recipients experiencing local reactions such as redness or tenderness at the site (Moss-Blundell *et al.,* 1981; Pivetaud *et al.,* 1986) and encephalitis being very rarely reported in young infants (Louis *et al.,* 1981; Stuart, 1956). Recent reports however have suggested that yellow fever vaccine, in rare instances, can cause illness that resembles infection with wild-type virus, especially in the elderly (Chan *et al.,* 2001; Martin *et al.,* 2001; Vasconcelos *et al.,* 2001).

Effectiveness of the vaccine in humans and animals has been attributed to the development of complement fixing antibodies to the NS1 protein and neutralizing antibodies to the E structural protein with a duration of protection as long as 35 years (Brandriss *et al.,* 1990; Lobigs *et al.,* 1987; Poland *et al.,* 1981). In animal studies, active immunization with the YFV structural protein E or the nonstructural protein NS1 or passive immunization with monoclonal antibodies to these proteins has been

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## TABLE 1

Yellow Fever Specific Proliferative Responses of PBMC from Yellow Fever Vaccine Recipients[a]

| Donor | Days postvaccination | Stimulation index[b] at indicated Ag dilution | |
|---|---|---|---|
| | | 1:80 | 1:160 |
| A[c] | 0 | 1.3 | 0.5 |
| | 7 | 0.1 | 0.7 |
| | 14 | 9.3 | 4.0 |
| | 28 | 10.6 | 8.1 |
| | 71 | 4.8 | 9.8 |
| B[c] | 0 | 1.3 | 3.5 |
| | 180 | 39.0 | 31.0 |
| C | 0 | 1.2 | 1.6 |
| | 56 | 376.0 | 237.0 |
| D | 0 | 2.2 | 0.9 |
| | 29 | 58.3 | 47.0 |

[a] PBMC ($2 \times 10^5$ cells) were incubated for 6 days in the presence of serial dilutions of yellow fever antigen. Cells were then pulsed with 1.25 uCi of [$^3$H]TdR for 8 h and thymidine incorporation was measured.

[b] Stimulation index (SI) is calculated as mean cpm of cultures with yellow fever antigen/mean cpm of culture with control antigen.

[c] These donors have received the inactivated Japanese encephalitis vaccine.

shown to protect mice and monkeys against lethal YFV infection (Brandriss *et al.,* 1986; Putnak and Schlesinger, 1990; Schlesinger *et al.,* 1986, 1993). The best evidence of the efficacy of the YFV vaccine in humans is based on epidemiological studies showing a decrease in incidence of YFV in affected areas after introduction of the vaccine (Durieux, 1956; Soper, 1938).

Our laboratory is interested in analyzing YFV-specific CD8$^+$ T cell responses in YFV vaccine recipients to better understand the role of T lymphocytes in protection after immunization. Strong evidence that CD8$^+$ T cells are important for viral clearance comes from murine models of influenza virus and LCMV infections (Doherty, 1996; Kuwano *et al.,* 1990). Human data in HIV, CMV, and EBV infections are also suggestive of an important role for virus-specific CD8$^+$ T cells (Cheynier *et al.,* 1992; Pinto *et al.,* 1995; Riddell *et al.,* 1992; Rooney *et al.,* 1995; Rowland-Jones *et al.,* 1998, 1993; Walter *et al.,* 1995). In this article, we characterize YFV-specific CTL responses in the peripheral blood mononuclear cells (PBMC) of four individuals who received the live attenuated YFV 17D vaccine. Proliferation and cytolytic responses to YFV were demonstrated in all individuals. We isolated 13 CD8$^+$ T cell lines, the majority of which were HLA-B35-restricted and directed against epitopes on the YFV structural protein E and the nonstructural proteins NS1, NS2b, and NS3. To our knowledge, this is the first report of YFV-specific human T cell responses.

## RESULTS

### Proliferative responses of PBMC to yellow fever antigen

To assess the induction of proliferation responses to YFV by vaccination, we collected PBMC from four healthy donors (A–D) prevaccination (Day 0) and at various time points postvaccination; Day 0 PBMC served as a negative control. These samples were thawed and then cultured with YFV antigen at various dilutions for 6 days and [$^3$H]thymidine incorporation was measured. The results are shown in Table 1. Day 0 PBMC from Donors A, C, and D showed no proliferation to yellow fever antigen (stimulation index ((SI) < 3), indicating that none of these donors had previous exposure to yellow fever virus. There was a low level of preexisting response to YFV antigen (SI = 1.3–3.5) in Day 0 PBMC of Donor B, which may be related to prior immunization with inactivated Japanese encephalitis vaccine. All four subjects developed proliferation responses to YFV (SI > 3) after vaccination; in three of four subjects SI values were >30. For Donor A, multiple postvaccination PBMC samples were tested in the same assay; proliferation responses to YFV were evident, compared to the donor's prevaccination PBMC, at 14 days postvaccination.

### Yellow fever specific cytolytic activity in bulk culture

YFV-specific cytolytic activity in postvaccination PBMC samples was measured 14 days after *in vitro* stimulation with infectious YFV using as target cells autologous BLCLs infected with recombinant vaccinia viruses expressing portions of the YFV genome. Results from these experiments are shown in Table 2. For Donors A and C, bulk culture CTL demonstrated much greater lysis of target cells infected with the vaccinia construct Vp729

## TABLE 2

Recognition of YFV Proteins by Bulk Culture Cytotoxic T Cells from Yellow Fever Vaccine Recipients[a]

| Donor (days postvaccination) | % Specific $^{51}$Cr release of target cells infected with[b] | | | |
|---|---|---|---|---|
| | Vp729 (E, NS1, NS2a, NS2b) | VAC3B (NS3 199–433) | VAC3C (NS3 398–623) | VAC control |
| A (71) | 65 | NT | NT | 11 |
| B (56) | 69 | 53 | 44 | 43 |
| C (55) | 61 | 11 | 11 | 13 |
| D (64) | 24 | 16 | 21 | 11 |

[a] PBMC obtained at the indicated time point postvaccination were stimulated with YFV on Day 0 and 7 of culture and tested on Day 14 of culture at an E/T ratio of 60:1. Results are derived from three separate experiments.

[b] YFV proteins encoded by recombinant vaccinia viruses Vp729, VAC3B, and VAC3C are shown in parentheses.

TABLE 3

Recognition of YF Proteins by CTLs Generated from PBMC of YF Vaccine Recipients[a]

| Donor | Cell line | % Specific $^{51}$Cr release of target cells infected with | | | | | | | Protein recognized |
| | | Vp729 (E, NS1, NS2a, NS2b) | Vp869 (PrM, E) | VAC3A (NS3 1−232) | VAC3B (NS3 199−433) | VAC3C (NS3 398−623) | Vp725 (NS1, NS2a) | Vp730 (E) | |
|---|---|---|---|---|---|---|---|---|---|
| A | A3.1 | <u>59</u> | −3 | NT | NT | NT | −4 | −9 | NS2b |
| | A3.2 | <u>63</u> | −3 | NT | NT | NT | 0 | −2 | NS2b |
| | A3.5 | <u>38</u> | 2 | NT | NT | NT | <u>100</u> | −4 | NS1/2a |
| | A3.14 | <u>68</u> | 2 | NT | NT | NT | <u>86</u> | −3 | NS1/2a |
| | A3.13 | <u>43</u> | <u>61</u> | NT | NT | NT | −4 | <u>76</u> | E |
| | B3.2 | −1 | NT | NT | <u>83</u> | −1 | NT | NT | NS3 |
| B | A3.7 | 3 | NT | NT | <u>22</u> | −1 | NT | NT | NS3 |
| | A10.29 | 1 | NT | NT | <u>38</u> | 2 | NT | NT | NS3 |
| | A10.52 | <u>46</u> | NT | 3 | 6 | −2 | NT | NT | nd[b] |
| C | A1.4 | <u>54</u> | NT | 2 | 2 | −1 | −3 | −1 | NS2b |
| | A10.39 | <u>22</u> | NT | <u>37</u> | 5 | −3 | −2 | −4 | Mixed |
| | A1.5 | 1 | NT | 0 | 1 | <u>60</u> | NT | NT | NS3 |
| D | A10.34 | −3 | NT | −3 | <u>54</u> | <u>59</u> | NT | NT | NS3 |

[a] Effector cells were added to autologous target cells infected with vaccinia recombinant viruses expressing portions of the yellow fever virus at an E/T ratio of 5 or 10. The T cell lines shown here were tested in separate experiments. Underlined values represent significant levels of lysis. NT, not tested.

[b] nd, not determined. This cell line was later found to recognize an epitope on the NS2b protein.

than target cells infected with a control vaccinia virus. Vp729 expresses the structural protein E and the nonstructural proteins NS1, NS2a, and NS2b. For Donor B, background lysis of target cells infected with control vaccinia virus was relatively high, but lysis of Vp729-infected target cells was significantly higher (69% vs 43%). Donor D PBMC showed a lower level of recognition of the E, NS1, NS2a, and NS2b proteins. Bulk culture CTL from Donors B and D showed only slightly higher lysis of target cells infected with recombinant vaccinia viruses expressing portions of the YFV NS3 protein than target cells infected with control vaccinia virus. These experiments demonstrate that cytotoxic T cell responses to YFV proteins (E, NS1, NS2a, or NS2b) can be detected in the PBMC of donors who received the YFV vaccine.

## Isolation of yellow fever specific CTL lines

To define epitopes recognized by YFV-specific CTL, we isolated YFV-specific T cell clones by limiting dilution. Wells positive for growth were screened for lysis of target cells infected with vaccinia recombinants expressing portions of the yellow fever genome. All YFV-specific CTL lines isolated were found to be CD8^{+} (data not shown). We characterized in detail 13 YFV-specific CTL lines derived from four donors (Table 3 and data not shown). We isolated other T cell lines that showed similar patterns of recognition as one or more of the cell lines shown.

Several YFV-specific CTL lines were isolated from the PBMC of Donor A obtained 10 weeks postvaccination. Cell lines A3.1 and A3.2 only recognized target cells that

expressed the nonstructural protein NS2b (Vp729) and failed to recognize target cells that only expressed either the structural proteins preM and E (Vp869 and Vp730) or the nonstructural proteins NS1 and NS2a (Vp725). Cell lines A3.14 and A3.5 only recognized target cells expressing NS1 or NS2a (Vp729 and Vp725). Cell line A3.13 only recognized target cells expressing the structural protein E (Vp729, Vp869, and Vp730). Cell line B3.2 was isolated from Donor A PBMC obtained 56 days postvaccination and only recognized target cells expressing amino acids 199−433 of the NS3 protein (VAC3B).

YFV-specific CTL lines were isolated from the PBMC of Donor B obtained 8 weeks postvaccination. Cell lines A3.7 and A10.29 only recognized target cells expressing amino acids 199−433 of the nonstructural protein NS3 (VAC3B). Cell line A10.52 recognized target cells infected with the recombinant vaccinia virus expressing the E, NS1, NS2a, and NS2b proteins (Vp729), but not target cells infected with recombinant vaccinia viruses expressing portions of the NS3 protein.

YFV-specific CTL lines were isolated from the PBMC of Donor C obtained 8 weeks postvaccination. Cell line A1.4 only recognized target cells expressing NS2b (Vp729). Cell line A1.5 only recognized target cells expressing amino acids 398−623 of the nonstructural protein NS3 (VAC3C). Cell line A10.39 recognized two vaccinia recombinants, Vp729 and VAC3A, suggesting that this CTL line was a mixture of two subpopulations, one recognizing NS2b and one recognizing amino acids 1−232 of NS3.

The one YFV-specific CD8^{+} T cell line that was isolated from the PBMC of Donor D obtained 2 months

TABLE 4

HLA Restriction of YFV-Specific T Cell Lines Isolated from Donor A[a]

| Target cell | HLA class I haplotypes | % specific lysis by indicated CD8^{+} T cell lines | | | | |
|---|---|---|---|---|---|---|
| | | A3.1 | A3.2 | A3.5 | A3.13 | A3.14 |
| Autologous | A3, A24, B35, Cw4 | 51 | 82 | 50 | 51 | 66 |
| Donor B | A1, <u>A24</u>, B44, <u>B35</u>, <u>Cw4</u> | 35 | 45 | 8 | 74 | 5 |
| CB | A2, A23, <u>B35</u>, <u>Cw4</u> | 49 | 64 | 45 | 44 | 61 |
| TG | A23, A29, B7, B44, <u>Cw4</u> | 5 | 1 | 7 | −5 | 1 |
| HMY 35.01 | B*3501 | 47 | 29 | 41 | 6 | 50 |
| HMY 35.02 | <u>B*3502</u> | 35 | 32 | 9 | 34 | 14 |
| HMY Control | | 2 | 3 | 2 | 1 | 1 |

[a] Effector cells were tested for recognition of autologous or partially HLA-matched allogeneic target cells infected with recombinant vaccinia virus Vp729 expressing the E, NS1, NS2a, and NS2b proteins. The T cell clones shown were tested in separate experiments. The HLA alleles shared with allogeneic target cells are underlined.

postvaccination, A10.34, recognized target cells infected with overlapping regions of the NS3 protein, VAC3B (aa 199–433) and VAC3C (aa 398–623), but not target cells infected with Vp729 or VAC3A, suggesting that the epitope recognized is in the region of amino acids 398–433 of the NS3 protein.

In summary, we isolated three CD8^{+} T cell lines that recognized the nonstructural protein NS2b, five CD8^{+} T cell lines (from four donors) that recognized the nonstructural protein NS3, two CD8^{+} T cell lines that recognized the nonstructural proteins NS1 or NS2a, and one CD8^{+} T cell line that recognized the structural protein E.

## HLA restriction of yellow fever specific CD8^{+} T cell lines

The HLA restriction of YFV-specific CD8^{+} T cell lines from two of the four YFV vaccine recipients (Donor A and B) was determined by testing each cell line against a panel of partially matched allogeneic BLCL infected with the vaccinia recombinant Vp729, which expresses the structural protein E and the nonstructural proteins NS1, NS2a, and NS2b. Results from five of the CD8^{+} T cell lines isolated from Donor A are shown in Table 4. All five CD8^{+} T cell lines recognized CB target cells, which shared HLA-B35 and HLA-Cw4, but none recognized TG target cells, which shared HLA-Cw4, suggesting that these T cell lines were HLA-35-restricted. However, only three of the five T cell lines recognized Donor B BLCL, which also shared HLA-B35. To explain these results, we tested these cell lines for recognition of Vp729-infected HMY cells stably transfected with either HLA B*3501 or B*3502. Although the parental HMY cells were previously reported to express low levels of HLA B*3503 (Zemmour et al., 1992), these cells were not recognized by any of the T cell lines. Cell lines A3.1 and A3.2 lysed HMY cells that expressed either HLA B*3501 or B*3502. Cell lines A3.5 and A3.14 only recognized the HMY target cells that expressed HLA B*3501, while cell line A3.14 only recog-

nized the HMY target cells that expressed HLA B*3502. Results of other experiments demonstrated that the Donor A T cell line B3.2 and the Donor B T cell lines A3.7 and A10.29 lysed only NS3 peptide-pulsed HMY cells that expressed HLA B*3502, while the Donor B T cell line A10.52 recognized NS2b peptide-pulsed HMY cells expressing either HLA B*3501 or B*3502 (data not shown).

## Analysis of Donor A and B HLA-B35 alleles

The results above showed that HLA-B35-restricted CD8^{+} T cell lines derived from the PBMC of Donors A and B recognized different HLA-B35 alleles. To better understand this result, we analyzed the HLA-B35 subtypes in these donors by direct sequencing of the PCR product of the HLA B genes from Donors A and B. This analysis showed that Donor B was homozygous for HLA B*3502. Direct sequencing of the PCR product of the HLA-B genes from Donor A suggested that Donor A was heterozygous for B*3502 and B*3503. To confirm this, we cloned the PCR product and determined the nucleotide sequence of four subclones. Three of the four subclones were B*3502 and one was B*3503. Restriction enzyme digestion was performed to confirm that the PCR product had two different sequences. HLA B*3502 cDNA has two NarI and two RsaI restriction enzyme sites, while HLA B*3503 cDNA has three NarI and one RsaI restriction enzyme sites. Digestion of the PCR product with NarI and RsaI followed by 3% agarose gel electrophoresis demonstrated the fragments expected from both cDNAs (data not shown). These results confirmed that Donor A was heterozygous for B*3502 and B*3503.

## Localization of CTL epitopes

To identify the specific peptides recognized by YFV specific CD8^{+} T cell lines, each cell line was tested for recognition of target cells pulsed with synthetic peptides. In the case of the NS2b and NS3 proteins, the 20-mer

TABLE 5

Summary of Epitopes Recognized by Human YFV-Specific
CD8$^+$ CTL Lines

| Donor | CTL line | Protein recognized | AA position of epitope | Sequence |
|---|---|---|---|---|
| A | A3.1 | NS2b | 110–118 | HPFALLLVL |
|   | A3.2 | NS2b | 110–118 | HPFALLLVL |
|   | A3.14 | NS1 | 356–365 | HAVPFGLVSM |
|   | A3.13 | E | 332–340 | IPVIVADDL |
|   | B3.2 | NS3 | 351–358 | TGHDWILA |
| B | A3.7 | NS3 | 349–358 | WNTGHDWILA |
|   | A10.29 | NS3 | 349–358 | WNTGHDWILA |
|   | A10.52 | NS2b | 110–118 | HPFALLLVL |

peptides overlapped by 10 amino acids and covered the sequence of the entire protein or, for NS3, that portion expressed by the recombinant vaccinia virus. For the E and NS1 proteins, we selected for synthesis 19–21-mer peptides that contained sequences of nine amino acids predicted to bind well to HLA B*3501. Once a peptide recognized by the CTL was identified, we mapped the epitope further using truncations of the peptide 9–12 amino acids in length. Results are summarized in Table 5.

Donor A T cell lines A3.1 and A3.2 recognized the 20-mer peptide corresponding to residues 101–120 of the NS2b protein (Fig. 1A) and efficiently recognized the predicted 9-mer within this region (residues 110–118, HPFALLLVL) at concentrations of ≥0.25 µg/ml. This 9-mer peptide was also recognized by the Donor B T cell line A10.52 (data not shown). The Donor A T cell line A3.13 recognized the 21-mer peptide corresponding to residues 329–349 of the E protein (Fig. 1B) and efficiently recognized the predicted 9-mer within this region (resi-
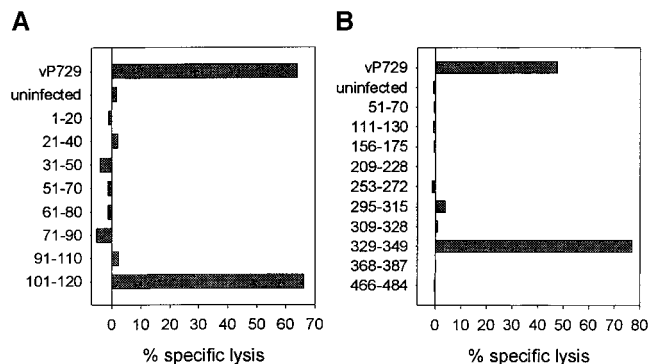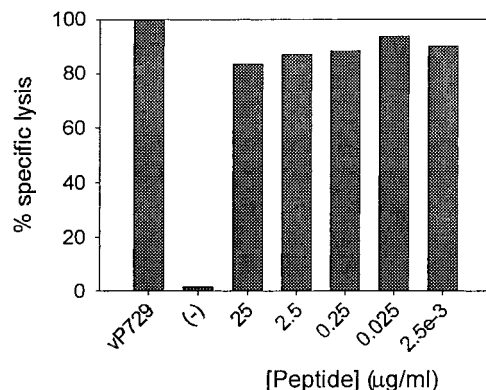


FIG. 2. Recognition of the optimal epitope on the YFV E protein by T cell line A3.13 from Donor A. Autologous target cells were infected with vP729 expressing the YFV E, NS1, NS2a, and NS2b proteins or were incubated with the indicated concentration of the YFV E (332–340) peptide (IPVIVADDL).

dues 332–340, IPVIVADDL) at concentrations of ≥0.0025 µg/ml (Fig. 2). The Donor A T cell line A3.14 recognized the 20-mer peptide corresponding to residues 351–370 of the NS1 protein (data not shown); this T cell line also recognized the smaller peptides NS1 (355–365) and NS1 (356–366), but did not recognize the peptide NS1 (357–368) (Fig. 3). These results demonstrate that the epitope recognized by T cell line A3.14 lies within residues 356–365 of NS1 (HAVPFGLVSM), with the N-terminal H residue necessary for recognition. The Donor A T cell line B3.2 and the Donor B T cell lines A3.7 and A10.29 recognized the 20-mer peptide corresponding to residues 341–360 of the NS3 protein (data not shown). The pattern of recognition of truncations of this peptide (Fig. 4) indicate that the epitope recognized by these T cell lines lies within residues 349–358 of NS3 (WNTGHD-WILA).

In summary, we identified CTL epitopes on the YFV E, NS1, NS2b, and NS3 proteins. Of note, the same epitopes on the NS2b and NS3 proteins were recognized by T cell lines derived from two HLA-B35 positive donors.



FIG. 1. Localization of the epitopes recognized by YFV-specific CD8+ T cell lines. (A) T cell line A3.1 from Donor A was tested for recognition of autologous target cells incubated with peptides derived from the sequence of the YFV NS2b protein. (B) T cell line A3.13 from Donor A was tested for recognition of autologous target cells incubated with peptides derived from the sequence of the YFV E protein. vP729 is the recombinant vaccinia virus expressing the YFV E, NS1, NS2a, and NS2b proteins.
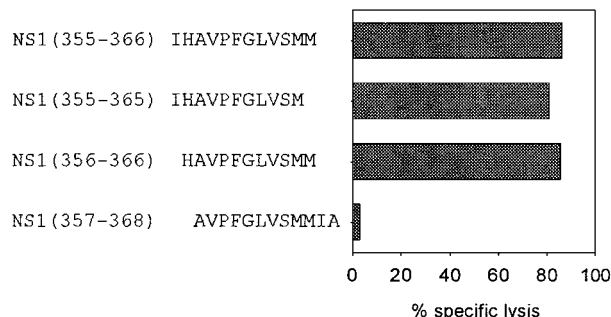


FIG. 3. Localization of the epitope on the YFV NS1 protein recognized by Donor A T cell line A3.14. Target cells were autologous BLCL incubated with the indicated peptide (25 µg/ml). The E/T ratio was 10.
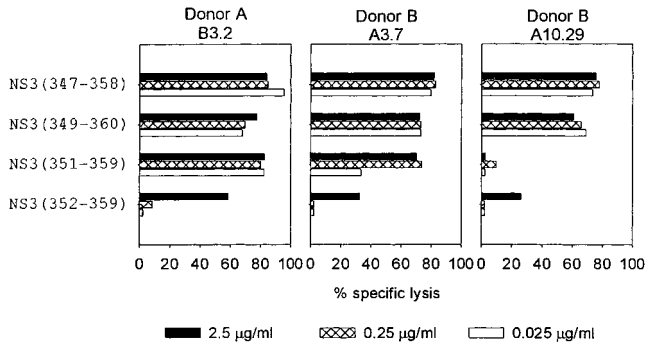
FIG. 4. Localization of the epitope on the YFV NS3 protein recognized by Donor A T cell line B3.2 and T cell lines A3.7 and A10.29 from Donor B. Target cells were autologous BLCL pulsed with the indicated peptide (25 μg/ml). The E/T ratio was 5.

## Kinetics of YFV specific T cell responses

We used the ELISPOT assay to quantitate interferon (IFNγ) production of YFV-specific CD8$^+$ T cells at the single cell level using thawed pre- and postvaccination PBMC from Donors A and B stimulated with the mapped E, NS1, NS2b, and NS3 epitopes (Fig. 5). Pre- and postvaccination PBMC were tested in the same assays.

For Donor A, no YFV specific T cells were detected at Day 0. The frequency of circulating T cells to the four mapped YFV epitopes after vaccination ranged between 1/2752 to less than 1/300,000 PBMC dependent on both the time point postvaccination and the epitope tested.

Responses to all four YFV epitopes were detected as early as 14 days postvaccination. The strongest responses were seen with the NS1 (355–366) peptide (1/2752–1/5357 PBMC) and the NS2b (110–118) peptide (1/4478–1/14,286 PBMC), and the frequency of T cells specific for these epitopes remained stable up to 19 months postvaccination. Both of these epitopes were more immunodominant than either the E (1/9677–1/50,000 PBMC) or the NS3 (1/25,000—less than 1/300,000 PBMC) epitopes.

For Donor B, there was an absence of YFV peptide specific T cells detected at Day 0 except to the NS2b epitope (1/150,000). We observed minimal or no T cell response to the NS1 epitope (<1/100,000) at any time point. Responses to the E and the NS2b epitopes were detected as early as 2 weeks postvaccination, whereas the response to the NS3 epitope was first detected at 1 month postvaccination. The responses to the E (1/7500–1/75,000 PBMC), NS2b (1/8695–1/150,000 PBMC), and NS3 (1/9677–1/75,000 PBMC) epitopes were similar over the time period tested, with a slight decline noted at 17 months postvaccination.

## DISCUSSION

We describe here the T cell responses to YFV in four volunteers who received the 17D live attenuated YFV vaccine. Bulk culture proliferation and cytolytic responses to YFV were detected in PBMC obtained post-
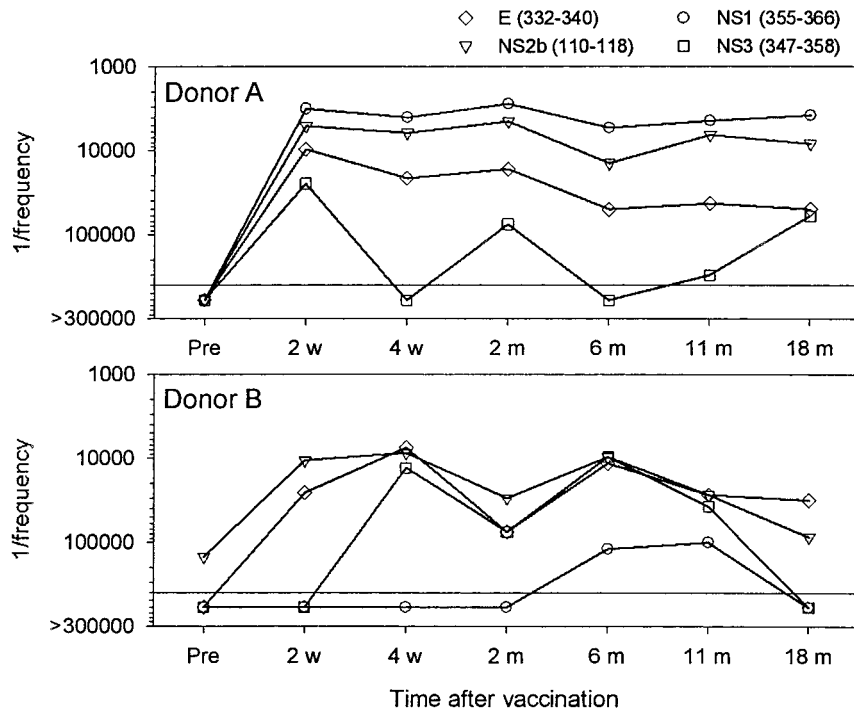


FIG. 5. Kinetics of antigen-specific T cell frequency up to 18 months after immunization with yellow fever vaccine in Donor A (top) and Donor B (bottom). PBMC from each of the donors were cultured for 18–20 h with the E, NS1, NS2b, or NS3 peptides at a concentration of 5 μg/ml and tested in a standard IFNγ Elispot assay. Each point represents the inverse frequency of antigen specific T cells to the indicated peptide.

vaccination in all four subjects studied. We characterized 13 YFV-specific CD8^{+} T cell lines and identified four HLA-B35-restricted T cell epitopes that were distributed on four different YFV proteins—E, NS1, NS2b, and NS3. Using the IFN$\gamma$ enzyme-linked immunospot (ELISPOT) assay, we examined the kinetics of the T cell response to these four epitopes in two HLA-B35+ subjects between 14 days and 19 months postvaccination. To our knowledge, this is the first description of human T cell responses to YFV and the first identification of CD8^{+} T cell epitopes on YFV proteins.

All of the T cell epitopes we defined were fully recognized in the context of HLA B35. HLA B35 is a relatively common allotype found in 10–28% of the population (Ragupathi et al., 1995). The peptide binding motif for HLA B*3501 and B*3503 consists of anchor residues at position 2 (P) and position 9 (F, M, or L) (Rammensee et al., 1995; Steinle et al., 1995). The characteristics of peptide binding to HLA B*3502 has not been described. The NS2b (HPFALLLVL) epitope fits the HLA B*3501/3503 binding motif and could be recognized by Donor A T cell lines in the context of B*3501 and B*3502. The E (IPVI-VADDL) epitope also fits the B*3501/3503 binding motif and was recognized by Donor A T cell line A3.13 in the context of B*3502. Although a 12-mer peptide recognized by the NS3-specific T cell clones (residues 347–358, EPWNTGHDWILA) contained a potential proline anchor residue, the mapped epitope lacked this residue. The NS1 epitope contains a proline residue, but our results show that this residue does not lie at position 2 of the peptide, since at least three residues N-terminal to the proline were required for recognition. For these peptides, other residues may contribute to the binding to HLA-B35. Schonbach et al. (1996) found that aliphatic hydrophobic side chains (Leu, Ile, Val, Met) at positions 3, 5, and 8 were found to enhance binding of 10-mer peptides to HLA B*3501. The 10-mer NS1 peptide contains hydrophobic valine residues at positions 3 and 8, and the 10-mer NS3 peptide (WNTGHDWILA) contains a hydrophobic isoleucine residue at position 8. The 8-mer NS3 peptide (TGHDWILA), which was recognized by some T cell lines, has other preferred residues for 9-mer peptides at positions 4 (D) and 6 (I), as well as a W residue at position 5 that has been noted in another HLA-B35-restricted T cell epitope (Koziel et al., 1992).

Several of the T cell clones isolated from Donor A recognized peptides presented by HLA B*3501, although this donor's genotype was HLA B*3502/B*3503; some of the T cell clones recognized peptides when presented by either HLA B*3501 or B*3502. These findings most likely reflect the subtle amino acid differences between the HLA B35 alleles. Sequence variations among the B35 subtypes are clustered in amino acid positions 94, 95, 97, 109, 114, and 166 (Beck et al., 1995; Ragupathi et al., 1995). The variations between the B*3501, B*3502, and B*3503 subtypes occur in amino acids 109, 114, and 116,

which lie in the $\beta$ sheet of the peptide binding pocket within the $\alpha$2 domain of the class I molecule. HLA B*3502 differs from B*3501 at positions 109, 114, and 116, while B*3503 differs from B*3501 only at position 116. These amino acids are involved in peptide side chain interactions in the C (97 and 114), D (114), E (97 and 114), and F (116) pockets (Ragupathi et al., 1995). Thus, the differences we observed in peptide recognition in the context of different HLA-B35 alleles likely reflect effects on peptide binding. The failure of Donor A NS1-specific T cell lines (A3.5 and A3.14) to recognize Donor B target cells (Table 4) suggests that this epitope can be presented by either HLA B*3501 or B*3503 but not by HLA B*3502 and is consistent with the observation that Donor B showed no significant T cell response to this epitope in ELISPOT assays (Fig. 5).

The IFN$\gamma$ ELISPOT assays showed the frequency of T cells specific for several of the HLA-B35-restricted T cell epitopes to be in the range of 1 in 10,000 PBMC or higher. T cell responses to the E and NS2b epitopes were prominent in both HLA-B35+ subjects studied, suggesting that these are important targets for the T cell response in HLA-B35+ individuals. The NS1 epitope was immunodominant in Donor A, but, as noted above, it was not recognized to any significant level in Donor B, suggesting that this epitope may be less relevant to the YFV-specific T cell response in the general population. T cell responses to the NS3 epitope were detected in both subjects, but were subdominant; this was somewhat surprising to us because the NS3 protein of dengue virus, a related flavivirus, appears to be a dominant target for the CD4^{+} and CD8^{+} T cell response (Livingston et al., 1995; Mathew et al., 1998; Zivny et al., 1999). Although both Donors A and B had received the inactivated Japanese encephalitis (JE) vaccine, we do not expect this to have altered our findings because inactivated vaccines are poor inducers of CD8^{+} T cell responses and would not be expected to contain nonstructural proteins. Prior studies have found no differences in replication of YFV 17D or antibody responses to YFV in vaccine recipients with previous exposure to JE virus (Sweet et al., 1962; Wisseman et al., 1962).

The frequency of YFV-specific T cells we detected is comparable to the frequency of T cells specific for CD8^{+} T cell epitopes on influenza A virus as measured by IFN$\gamma$ ELISPOT assays (Jameson et al., 1999). However, in the case of influenza virus there is a high likelihood of repeated exposure that may boost virus-specific T cells. Most other data on the frequency of virus-specific CD8^{+} memory T cells has related to persistent viral infections such as HIV, EBV, and CMV, where virus-specific T cells make up as much as 3–5% of all circulating CD8^{+} T cells after the acute phase of infection (Callan et al., 1998; Dalod et al., 1999a,b; Kern et al., 1998; Tan et al., 1999). Data on nonrecurring acute viral infections is much more limited; one study using intracellular IFN$\gamma$ staining re-

ported that measles virus-specific T cells represented a median of 0.24% of circulating CD8$^+$ T cells in healthy adults decades after the original infection (Nanan et al., 2000).

Because exposure to YFV is geographically limited, immunization with the YFV 17D vaccine may provide an ideal system to study the kinetics of the human T cell response to an acute virus infection. We found that YFV peptide-specific T cells were induced as early as 14 days postvaccination and that the frequency of these T cells was maintained at a relatively stable level for up to 18 months postvaccination. The kinetics of the T cell response to acute virus infections have been well characterized in laboratory mice, including to other flaviviruses. For example, van der Most et al. (2000) studied the T cell response to a YFV-dengue virus chimera in BALB/c mice using intracellular IFN$\gamma$ staining; memory CD8$^+$ T cells specific for an epitope on the dengue E protein represented 1/700 CD8$^+$ T cells at 100 days after immunization. In contrast, there have been few studies that have examined the kinetics of the memory T cell response to immunization or natural infection in humans. Pathan et al. (2000) reported that the CD8$^+$ T cell response to a Mycobacterium tuberculosis ESAT6 epitope remained stable over a 2-year period in an asymptomatic household contact. Rahman et al. (2000) assessed the T cell response to pooled hepatitis B peptides at various time points up to 32 weeks after vaccination with the recombinant hepatitis B vaccine. They detected a transient increase in peptide-specific T cell frequencies 2 weeks after booster immunization that was not sustained. Our group has found increases in the frequency of influenza A virus peptide-specific T cells at 14 and 56 days after receipt of an adjuvanted influenza vaccine (unpublished data); longer term follow-up was not available.

There is no data to permit a comparison between the vaccine-induced T cell responses to YFV we have measured with the T cell response to infection with wild-type YFV. Furthermore, there is no data to establish the role of vaccine-induced T cell responses in the protective efficacy of the YFV 17D vaccine. However, the four CTL epitopes we have identified are well conserved among isolates of YFV. For the E epitope, sequence data are available for comparison from over 20 viruses (Chang et al., 1995); the sequence is fully conserved in all isolates from genotypes I and IIA, whereas there is a conservative substitution of M for I at position 4 in the epitope in isolates from genotype IIB. For the other epitopes, only four to five wild-type YFV sequences are available for comparison. The sequences of the NS1 and NS3 epitopes are fully conserved. The sequence of the NS2b epitope is fully conserved in three of the published sequences, while there is a single nonconservative substitution of S for F at position 3 in a strain of YFV isolated from a patient from Cote d'Ivoire in 1982 (strain 85-82H, GenBank Accession No. U54798).

In vivo cell depletion experiments by van der Most et al. (2000) demonstrated a role for both CD4$^+$ and CD8$^+$ T cells in the protective efficacy of the YFV/dengue chimera against intracerebral challenge with dengue virus infection in mice. Mice immunized with a DNA vaccine against influenza A virus were protected against lethal challenge when the frequency of NP epitope-specific T cells was greater than 1/100,000 spleen cells (Fu et al., 1999). One group estimated the circulating frequency of CTL in Gambian prostitutes who remained seronegative despite multiple exposures to HIV at 1/3200–1/50,000, offering some correlate of protection from illness (Rowland-Jones et al., 1998). These data, along with the evidence of sequence conservation with wild-type YFV strains, support the hypothesis that the CD8$^+$ T cell responses to YFV 17D vaccine we observed could contribute to protection against illness.

Given the conserved features of flavivirus genome organization and the safety and effectiveness of the YFV vaccine, there has been interest in developing vaccines against other flavivirus infections, such as dengue and Japanese encephalitis, based on live attenuated chimeric viruses using the YFV genetic backbone (Guirakhoo et al., 2000; van Der Most et al., 2000). An improved understanding of immune responses induced by the YFV vaccine will help us understand how preexisting immunity to the YFV genetic component might alter the effectiveness of these chimeric vaccines.

## MATERIALS AND METHODS

### PBMC donors

Blood was obtained from four healthy individuals who had received the live attenuated 17D yellow fever vaccine after informed consent was obtained. Donors A and B received the inactivated Japanese encephalitis vaccine 14 months after and 4 years prior to the administration of the yellow fever vaccine, respectively. Donor A PBMC were collected prevaccination and at the following time points postvaccination—7 days, 14 days, 4 weeks, 10 weeks, 7 months, 11 months, 15 months, and 19 months. Donor B PBMC were collected prevaccination and at the following time points postvaccination—7 days, 12 days, 4 weeks, 8 weeks, 6 months, 10 months, and 17 months. Donor C PBMC were collected prevaccination and at 8 weeks and 6 months postvaccination. Donor D PBMC were collected prevaccination and at the following time points postvaccination—4 weeks, 9 weeks, and 6 months. The HLA class I alleles of these donors are as follows: Donor A—A3, A24, B35, Cw4; Donor B—A1, A24, B35, Cw4; Donor C—A2, A11, B40, Cw3, Cw7; Donor D—A2, A68, B27, B40, Cw2. PBMC were purified by Ficoll–Hypaque density gradient centrifugation and cryopreserved until needed.

## B lymphoblastoid cell lines (BLCL)

PBMC ($1–4 \times 10^6$) were thawed and then cultured in RPMI 1640 supplemented with penicillin, streptomycin, L-glutamine, HEPES, and 20% FBS in the presence of a 1:3 dilution of EBV from B95-8 cells (American Type Culture Collection) in 24-well flat bottom plates (Becton Dickinson, Franklin Lakes, NJ) as previously described (Bukowski *et al.,* 1989). Cyclosporine A was added at 1 $\mu$g/ml.

## Proliferative responses of PBMC to viral antigen

PBMC ($1 \times 10^5$) were thawed and then cultured with inactivated YFV 17D antigen or control antigen, which were prepared in Vero cells as described (Kurane *et al.,* 1989b), at various dilutions in 0.2 ml AIM V medium (GIBCO-BRL) containing 10% human AB serum (NABI, Boca Raton, FL) in 96-well round-bottom microtiter plates (Becton Dickinson) at 37°C for 6 days. The cells were pulsed with 1.25 $\mu$Ci [³H]thymidine for 8 h at 37°C before they were harvested using a multiharvester (Skatron Inc., Sterling, VA). [³H]thymidine incorporation was measured in a liquid scintillation counter (1205 Betaplate; Pharmacia, Wallac Oy, Finland). Stimulation index was calculated as follows: mean cpm of cultures with viral antigen/mean cpm of cultures with control antigen.

## *In vitro* stimulation of PBMC

PBMC obtained between 8 and 10 weeks postvaccination ($2–5 \times 10^6$ per well) were thawed and then resuspended in 1.5 ml of AIMV containing 15% human AB serum in a 24-well plate (Becton Dickinson). YFV (YF-VAX, Connaught Laboratories) was propagated in Vero cells and the infectious virus stock was added to PBMC at a 1:160 final dilution. Recombinant human IL-2 (20 U/ml) was added on Day 4 or 5 in 1 ml fresh AIMV containing 10% human AB serum and the medium was replenished every third day thereafter. Bulk culture ⁵¹Cr release assays were performed between Days 7 and 14 of culture. PBMC were restimulated on Day 7 with infectious YFV ($1 \times 10^8$ IU/ml) and $5 \times 10^6$ gamma-irradiated autologous PBMC.

## CTL lines

YFV-specific CTL lines were established by limiting dilution plating as previously described (Kurane *et al.,* 1989a). Briefly, PBMC stimulated *in vitro* with infectious YFV, as described above, were plated at 1, 3, 10, or 30 cells/well in 96-well round bottom plates (Becton Dickinson) in 0.2 ml of AIMV-15% FBS with 0.1 $\mu$g/ml anti-CD3 antibody (12F6; a gift of Dr. Johnson Wong, MGH), 50 U/ml rhIL-2, and $1 \times 10^5$ gamma-irradiated allogeneic PBMC. Medium was replaced every 3 days and wells were restimulated with anti-CD3 and gamma-irradiated feeder cells every 14 days. Individual wells were tested
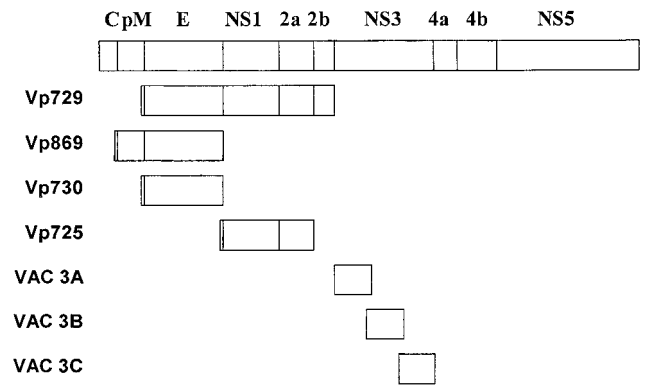


FIG. 6. Schematic of the recombinant vaccinia viruses containing portions of the yellow fever genome used in our experiments.

for recognition of target cells infected with recombinant vaccinia viruses expressing YFV proteins in a ⁵¹Cr release assay, and cells in positive wells were expanded and restimulated as described above. Surface expression of CD8 was determined by flow cytometry with fluorescein isothiocyanate-conjugated antibodies (Becton–Dickinson).

## HMY cell lines

Hmy2.C1R (Hmy) is a human plasma cell line that lacks endogenous HLA A and B antigens (Storkus *et al.,* 1987). Hmy lines transfected with B35.01 or B35.02 were generated as previously described (Ooba *et al.,* 1989) and were kindly provided by Masafumi Takaguguchi (University of Tokyo, Tokyo, Japan).

## Recombinant vaccinia viruses

Recombinant vaccinia viruses (Vp729, Vp869, Vp725, Vp730) expressing YFV proteins were kindly provided by J. Tartaglia, Virogenetics Corp., Troy, NY (Pincus *et al.,* 1992). Vp729 expresses the 19 aa signal sequence preceding the N terminus of E as well as the structural protein E and the nonstructural proteins NS1, NS2a, and NS2b. Vp869 expresses the 21 aa signal sequence preceding the N terminus of the structural protein precursor prM as well as prM and E proteins. Vp725 expresses the 17 aa signal sequence preceding the N terminus of nonstructural protein NS1 and the nonstructural protein NS1 and NS2a. Vp730 expresses the 19 aa signal sequence preceding the N terminus of the E protein as well as the structural protein E. VAC3A, VAC3B, and VAC3C were made by our laboratory using methods previously described (Saikh *et al.,* 1993). VAC3A expresses amino acids 1–232 of the nonstructural protein NS3. VAC3B expresses amino acids 199–433 of the nonstructural protein NS3. VAC3C expresses amino acids 398–623 of the nonstructural protein NS3. A schematic of the recombinant vaccinia viruses used in this study is shown in Fig. 6.

## Synthetic peptides

Peptides corresponding to YFV sequences were synthesized at the Protein Chemistry Core Facility at the University of Massachusetts Medical School with an automated Rainin Symphony peptide synthesizer. Peptides varied from 8 to 20 amino acids in length. For the nonstructural proteins NS2b and NS3, the synthetic peptides were originally 20 amino acids in length and overlapped by 10 amino acids to encompass the entire protein. Smaller peptides were subsequently produced to better define the epitopes. 20-mer peptides for the structural protein E and the nonstructural protein NS1 were selected for synthesis on the basis of the results of an algorithm to predict HLA-peptide binding (http://bimas. dcrt.nih.gov).

## Preparation of target cells

*Virus-infected targets.* BLCL ($3 \times 10^5$) were infected with recombinant vaccinia virus at a multiplicity of infection of 15 for 60 min at 37°C. The cells were then diluted in 1 ml of RPMI 1640 containing 10% FBS for an additional 12–16 h. Target cells were then labeled with 0.25 mCi of $^{51}$Cr for 60 min at 37°C. Following labeling, the cells were washed three times and resuspended at $1.5 \times 10^4$/ml in RPMI containing 10% FBS for use in cytotoxicity assay. Autologous and partially HLA-matched allogeneic BLCL were used as target cells in the assays.

*Peptide pulsed targets.* Uninfected BLCL were $^{51}$Cr labeled as described above. Labeled cells were incubated with 25 $\mu$g/ml of peptide in 96-well round bottom plates for 30 min at 37°C before the addition of effector cells. The peptides remained in the wells for the duration of the assay.

## $^{51}$Cr release assay

Effector cells were added to $1.5 \times 10^3$ $^{51}$Cr-labeled target cells at various effector cell/target cell (E/T) ratios. Plates were incubated for 4.5 h at 37°C; supernatants were harvested (Supernatant Collection System, Skatron Instruments) and counted in a Hewlett–Packard $\gamma$ counter. Specific lysis was calculated as $100 \times$ (experimental release − spontaneous release)/(maximum release − spontaneous release). All assays were performed in triplicate. Negative controls included target cells infected with wild-type vaccinia virus and uninfected or unpulsed target cells.

## Enzyme-linked immunospot assay for single cell IFN$\gamma$ secretion

ELISPOT assays were performed as described previously (Lalvani *et al.,* 1997). Briefly, 96-well filtration plates (MAIP S45; Millipore, Bedford, MA) were coated with 15 $\mu$g/ml of mouse anti-human IFN$\gamma$ monoclonal antibody (NIB42 hybridoma) (Pharmingen, San Diego, CA). After washing, RPMI containing 10% FBS was added at 100 $\mu$l/well for 2 h at 37°C. PBMC were thawed and then resuspended in RPMI containing 10% FBS at $3 \times 10^5$ cells/well. Peptides were added at a final concentration of 5 $\mu$g/ml with plates incubated for 18–20 h at 37°C. Plates were washed with PBS and then with PBS containing 0.05% Tween. Biotinylated mouse anti-human IFN$\gamma$ monoclonal antibody (4S.B3 antibody) (Pharmingen) was then added at a final concentration of 2 $\mu$g/ml and plates were incubated at room temperature for 2 h, followed by addition of 1:400 dilution of avidin peroxidase (Sigma) at 100 $\mu$l/well. Plates were then incubated for 45 min at room temperature. Substrate (3-amino-9-ethyl-carbazole-.15% $H_2O_2$; Sigma) was added and left for 10 min at room temperature. The precursor frequency was calculated as (number of spots in wells containing peptide − number of spots in wells with medium alone)/(total number of cells per well). The number of spots in the negative control wells (medium alone) ranged from 2 to 15. Experiments were performed in duplicate.

## Determination of HLA B35 subtypes

Total RNA was isolated from BLCL using the Ultraspec RNA Isolation System (Biotec Laboratories, Houston, TX). To synthesize the cDNA, 2 $\mu$g of total RNA was added to the reaction mixture containing 50 mM Tris–HCl (pH 8.3 at 25°C), 75 mM KCl, 3 mM MgCl$_2$, 10 mM DTT, 0.5 mM dNTPs, 100 units of RNAguard (Pharmacia Biotech, Piscataway, NJ), 0.5 $\mu$g pd(T)$_{12-18}$ (Pharmacia Biotech), and 500 units of M-MLV Reverse Transcriptase (Promega, Madison, WI) in 100 $\mu$l. The RT reaction was carried out at 37°C for 60 min followed by heat inactivation at 95°C for 10 min. HLA-B genes were amplified by an HLA-B specific primer set: upstream primer (5P2T): 5′-GGG CGT CGA CGG ACT CAG AAT CTC C$\underline{T}$C AGA CGC CGA G-3′ (underlined base is different from original primer HLA-5P2 (27)), and downstream primer (3B): 5′-CCG CAA GCT TCT GGG GAG GAA ACA CAG GTC AGC ATG GGA AC-3′ (Zemmour *et al.,* 1992). Primers 5P2T and 3B have *Sal*I and *Hin*dIII restriction enzyme sites for cloning, respectively. PCR was performed using the GeneAmp XL PCR Kit (PE Applied Biosystems, Foster City, CA). One microliter of cDNA was added to the PCR reaction mixture containing 1× XL buffer II, 0.2 mM dNTPs, 0.8 mM Mg(OAc)$_2$, 0.25 pmol/$\mu$l of each primer, and 2 u of rTth DNA polymerase, XL in 100 $\mu$l. PCR was performed in a DNA Thermal Cycler 480 (PE Applied Biosystems) at 94°C for 60 s, followed by 30 cycles at 94°C for 30 s and 65°C for 5 min, and finally 1 cycle at 72°C for 10 min. The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA), digested with the restriction enzymes *Sal*I and *Hin*dIII (Promega), and ligated to the pBluescript II SK (+) vector (which was also cut by *Sal*I and *Hin*dIII) using the Fast-Link DNA Ligation Kit (Epicentre Technologies, Madison, WI) following the

manufacturer's recommendation. The ligation mixture was used to transform *Escherichia coli* INVαF′ (Invitrogen, Carlsbad, CA). Colonies were picked up, grown in LB media, and used to prepare plasmid DNA (Sambrook *et al.,* 1989).

DNA templates for sequencing were purified using the QIAquick PCR Purification Kit or QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer's recommendations. Sequencing was carried out using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit and the ABI PRISM 377 DNA Sequencer (PE Applied Biosystems) at the University of Massachusetts Medical School Nucleic Acid Facility. Primers used for the sequencing reaction were 2S (5′-AGG GGC CGG AGT ATT GGG AC-3′), 3S (5′-CGG CAA GGA TTA CAT CGC CCT G-3′), and 3N (5′-CAG GGC GAT GTA ATC CTT GCC G-3′), which cover the coding regions of the α1, α2, and α3 domains of the HLA-B molecule (Ennis *et al.,* 1990).

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