JOURNAL OF VIROLOGY, May 2004, p. 5216–5222 Vol. 78, No. 10
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.10.5216–5222.2004

A Mutation in the HLA-B*2705-Restricted $NP_{383–391}$ Epitope Affects the Human Influenza A Virus-Specific Cytotoxic T-Lymphocyte Response In Vitro

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Received 13 November 2003/Accepted 16 January 2004

Viruses can exploit a variety of strategies to evade immune surveillance by cytotoxic T lymphocytes (CTL), including the acquisition of mutations in or adjacent to CTL epitopes. Recently, an amino acid substitution (R384G) in an HLA-B*2705-restricted CTL epitope in the influenza A virus nucleoprotein (nucleoprotein containing residues 383 to 391 [$NP_{383–391}$]; S*R*YWAIRTR, where *R* is the residue that was mutated) was associated with escape from CTL-mediated immunity. The effect of this mutation on the in vitro influenza A virus-specific CTL response was studied. To this end, two influenza A viruses, one with and one without the $NP_{383–391}$ epitope, were constructed by reverse genetics and designated influenza viruses A/NL/94-384R and A/NL/94-384G, respectively. The absence of the HLA-B*2705-restricted CTL epitope in influenza virus A/NL/94-384G was confirmed by using ^{51}Cr release assays with a T-cell clone specific for the $NP_{383–391}$ epitope. In addition, peripheral blood mononuclear cells (PBMC) stimulated with influenza virus A/NL/94-384G failed to recognize HLA-B*2705-positive target cells pulsed with the original $NP_{383–391}$ peptide. The proportion of virus-specific $CD8^+$ gamma interferon (IFN-γ)-positive T cells in in vitro-stimulated PBMC was determined by intracellular IFN-γ staining after restimulation with virus-infected autologous B-lymphoblastoid cell lines and C1R cell lines expressing only HLA-B*2705. The proportion of virus-specific $CD8^+$ T cells was lower in PBMC stimulated in vitro with influenza virus A/NL/94-384G obtained from several HLA-B*2705-positive donors than in PBMC stimulated with influenza virus A/NL/94-384R. This finding indicated that amino acid variations in CTL epitopes can affect the virus-specific CTL response and that the $NP_{383–391}$ epitope is the most important HLA-B*2705-restricted epitope in the nucleoprotein of influenza A viruses.

Cytotoxic T lymphocytes (CTL) play an important role in the control of viral infections, including those caused by influenza viruses. It has been shown that in mice, CTL contribute to protective immunity against influenza viruses of various subtypes (25). In addition, influenza A virus-specific CTL in humans were found to reduce virus titers in the lungs and morbidity upon infection (27). To evade host CTL responses, viruses have developed a variety of mechanisms to prevent recognition by specific CTL (24, 30). These mechanisms involve major histocompatibility complex (MHC) class I downregulation, as described for the human immunodeficiency virus type 1 Nef protein (11, 40) and the adenovirus E3/19K protein (8), and blocking of antigen presentation of viral epitopes, as described for certain herpesvirus proteins (22, 23). Alternatively, viruses can accumulate in or adjacent to CTL epitopes mutations that affect peptide processing and presentation, binding to MHC class I molecules, and/or recognition by specific T cells (20, 45). This escape mechanism has been described predominantly for chronic virus infections, such as those caused by lymphocytic choriomeningitis virus (29, 32), Epstein-Barr virus (1, 9, 13, 14, 17), human immunodeficiency virus (7, 12, 18, 19, 24, 28, 31, 33, 34), hepatitis B virus (2, 3), and hepatitis C virus (10, 44).

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Recently, amino acid sequence variations in CTL epitopes in the nucleoprotein (NP) of influenza A viruses were identified (5, 41). In the HLA-B*3501-restricted epitope $NP_{418–426}$ (NP containing residues 418 to 426), variations were observed in the T-cell receptor contact residues (5). These variants emerged in a chronological order, and CTL specific for older variants failed to recognize the mutated versions of the same epitope. In addition, an amino acid sequence variation was observed at position 384 of the NP. An arginine at this position is an anchor residue for the HLA-B*0801-restricted epitope $NP_{380–388}$ (ELRSRYWAI) and the HLA-B*2705-restricted epitope $NP_{383–391}$ (SRYWAIRTR) (21). The observed R384G mutation resulted in the loss of the anchor residue and, as a result, abolished recognition by CTL (37, 41). Although the rapid fixation of the mutation was explained by small selective advantages and population dynamics in a theoretical model (16), it is unclear to what extent a single amino acid substitution in a CTL epitope affects the overall virus-specific CTL response in humans.

In this study, this issue was addressed in vitro by using peripheral blood mononuclear cells (PBMC) from HLA-B*2705-positive individuals and genetically engineered recombinant influenza viruses containing an arginine or a glycine at position 384 (but otherwise identical) and therefore possessing or not possessing the HLA-B*2705-restricted epitope $NP_{383–391}$ (SRYWAIRTR). Using these viruses for the stimulation of PBMC, we assessed the in vitro CTL response. It was found

that the R384G substitution significantly impaired the influenza virus-specific CTL response in vitro.

MATERIALS AND METHODS

Plasmids and site-directed mutagenesis. For the generation of recombinant influenza viruses, RNA was extracted from culture supernatants containing influenza virus A/Netherlands/18/94 (A/NL/18/94) by using a High Pure RNA isolation kit (Roche Diagnostics GmbH, Mannheim, Germany). The RNA was used in a single-tube reverse transcription-PCR to amplify viral NP segments. After annealing of the primers AGCAAAAGCAGGGT and AGTAGAAACA AGGGTATTTTTC, first-strand synthesis was carried out with 50-μl volumes of 20 mM Tris-HCl buffer (pH 8.8) containing 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.1 mg of bovine serum albumin (BSA)/ml, 10 mM deoxynucleoside triphosphates, 10 mM dithiothreitol, RNasin, 5 IU of Superscript II reverse transcriptase, and 5 IU of *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, Calif.). After incubation for 45 min at 42°C, the mixture was heated at 95°C for 3 min, followed by 40 cycles of denaturation (1 min at 95°C), annealing (2 min at 37°C), and elongation (3 min at 72°C). For the addition of BsaI restriction sites, an additional amplification of 30 cycles was performed with the primers CTAGGTCTCTTATTAGTAGAAACAAGG and GGGAGGTCTCCGGCCAGCAAAAGCAGG (underlining indicates restriction sites). The amplicon was purified by electrophoresis on agarose gels according to standard methods and inserted between the human polymerase I promoter and the hepatitis delta ribozyme sequence of plasmid pSP72-PhuThep (15).

For site-directed mutagenesis, the coding sequence of the NP gene of influenza virus A/NL/18/94 was amplified by PCR with the primers CAGCGGCCG CATGGCGTCCCAAGGC and CACTCGAGTTAATTGTCGTACTCCT CTGC and cloned into pBluescript (Stratagene) after digestion with NotI and XhoI. Using this plasmid as a template, site-directed mutagenesis was performed by PCR in order to obtain an arginine at position 384 instead of a glycine (G384R) as previously described (39). The mutated sequence was exchanged by using restriction site SphI in the NP gene and restriction site XhoI in pBluescript, 3′ of the insert. Subsequently, the SacI fragment with the mutation at position 384 was exchanged for SacI fragments in genomic constructs of the NP gene of influenza A virus A/NL/18/94. The nucleotide sequences of all cloned NP genes were determined by using standard procedures as previously described (41) in order to confirm the identity of the sequences.

Plasmid pHMG-NP, from which the NP of influenza virus A/PR/8/34 was transcribed, was kindly provided by P. Palese. The bidirectional reverse genetics plasmids pHW181 through pHW188 for the transcription of viral gene segments of influenza virus A/WSN/33 were kindly provided by R. G. Webster.

Generation of viruses. The unidirectional plasmid of the genomic construct of the NP of A/NL/18/94 was transfected into 293T cells with the bidirectional constructs containing the PB1, PB2, PA, HA, NA, M, and NS gene segments of A/WSN/33 and pHMG-NP expressing the NP of PR/8/34. For this purpose, 10^6 293T cells were cultured overnight in Dulbecco minimal essential medium (Cambrex, East Rutherford, N.J.) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 100 IU of penicillin/ml and 100 μg of streptomycin/ml (antibiotics) in gelatin-coated 10-cm petri dishes. The cells then were transfected with 5 μg of each of the plasmids by the calcium phosphate precipitation method as described previously (15). After 24 h, the cells were washed with phosphate-buffered saline (PBS), and 10 ml of Dulbecco minimal essential medium containing 2% FCS was added. After another 24 h, the culture supernatants of the transfected 293T cells were harvested, and infectious virus titers were determined as previously described (36). Titers of 10^3 50% tissue culture infective doses/ml were obtained routinely with the A/NL/94-384G and A/NL/94-384R constructs. Subsequently, virus stocks were prepared by infecting confluent Madin-Darby canine kidney (MDCK) cells in 162-cm^2 flasks with 1 ml of supernatant from the transfected 293T cells in 4 ml of Eagle minimal essential medium (Cambrex) supplemented with 4% BSA, 0.02 M HEPES, 0.01 M $NaHCO_3$, antibiotics, and 0.1% trypsin (infection medium) at 37°C for 1 h. The cells were washed once with PBS and then cultured at 37°C in infection medium. After 3 days, culture supernatants were harvested, cleared by low-speed centrifugation, divided into aliquots, and stored at −70°C until use. Viruses with the $NP_{383–391}$ epitope and viruses without the $NP_{383–391}$ epitope were designated A/NL/94-384R and A/NL/94-384G, respectively. Upon infection of MDCK cells, virus titers were amplified up to 10^7 50% tissue culture infective doses/ml for both viruses.

PBMC. PBMC from healthy HLA-B*2705-positive blood donors were isolated from heparinized blood (Sanquin Bloodbank, Rotterdam, The Netherlands) by density gradient centrifugation with lymphocyte separation medium (ICN Biomedicals/Cappel, Aurora, Ohio) and cryopreserved at −135°C. Genetic subtyping was performed in the Laboratory for Histocompatibility and Immunogenetics at the Sanquin Bloodbank by using a commercial typing system (GenoVision, Vienna, Austria).

In vitro stimulation of PBMC with influenza A viruses. PBMC were resuspended in RPMI 1640 medium containing 25 mM HEPES buffer and L-glutamine (Cambrex) and supplemented with 10% FCS and antibiotics (R10F). Five million PBMC were infected with influenza virus A/NL/94-384G, A/NL/94-384R, or Resvir-9, a reassortant of strains A/Puerto Rico/8/34 (H1N1) and A/Nanchang/933/95 (H3N2) containing the NP, HA, and NA of A/Nanchang/933/95. A multiplicity of infection of 3 in a volume of 5 ml of R10F was used as described previously (4). After 1 h at 37°C, the cells were resuspended in RPMI 1640 medium supplemented with 10% human AB serum (Sanquin Bloodbank), antibiotics, and 20 μM β-mercaptoethanol and added to noninfected PBMC at a ratio of 1:1 in a 25-cm^2 culture flask. After 48 h of stimulation, recombinant interleukin 2 was added to a final concentration of 50 IU/ml. After an additional 7 to 8 days, the cells were used as effector cells in ^{51}Cr release assays and for the enumeration of virus-specific $CD8^+$ T cells by intracellular gamma interferon (IFN-γ) staining (see below).

$CD8^+$-T-cell clones. The generation of a $CD8^+$-T-cell clone directed against the HLA-B*2705-restricted epitope $NP_{383–391}$ derived from the NP was described previously (41). A $CD8^+$-T-cell clone directed against the immunodominant and conserved HLA-A*0201-restricted epitope $M1_{58–66}$ derived from the matrix protein (M1) was established by similar procedures.

Isolation of $CD8^+$ T cells. $CD8^+$ T cells were isolated from effector cell populations by magnetic sorting with a $CD8^+$ cell selection kit (Dynal Biotech GmbH, Hamburg, Germany). The cells were washed once with PBS supplemented with 2% FCS (P2F) and subsequently resuspended in P2F at a concentration of 10^7/ml. Capture Dynabeads were added to the cell suspension at a Dynabead/$CD8^+$-T-cell ratio of 8:1. After 30 min of incubation on ice, the mixture of Dynabeads and cells was washed five times with 5.0 ml of P2F. The Dynabeads, together with the attached cells, were reconstituted in 200 μl of RPMI 1640 medium supplemented with 1% FCS. To detach the cells from the Dynabeads, 20 μl of DETACHaBEAD (Dynal Biotech) was added. After 1 h of incubation at 20°C, the released cells were isolated, washed once with R10F, and used as effector cells in ^{51}Cr release assays with C1R cells as target cells.

Target cells. Autologous B-lymphoblastoid cell lines (BLCL), produced as described previously (38), and two C1R cell lines, kindly provided by P. Romero (C1R cell line) and J. Lopez de Castro (HLA-B*2705-transfected C1R cell line [C1R-B27]), were used as target cells. Peptide labeling was performed by incubating 10^6 cells overnight with 5 μM peptide in 1 ml of R10F. Peptides were manufactured, purified by high-pressure liquid chromatography, and analyzed by mass spectrometry (Eurogentec, Seraing, Belgium). For exogenous protein labeling, 50 μg of recombinant influenza virus NP (rNP), derived from influenza virus A/HK/2/68 (rNP-HK) or A/NL/18/94 (rNP-NL), was added to 10^6 cells in 1 ml of R10F as described previously (42). For infection with influenza virus A/NL/94-384G and A/NL/94-384R, target cells were infected at a multiplicity of infection of 3 in a volume of 1 ml. After incubation for 1 h at 37°C, the cells were resuspended in R10F and incubated for 16 to 18 h. BLCL and C1R cells were equally susceptible to infection with influenza virus A/NL/94-384G and A/NL/94-384R, as determined by immunofluorescence assays with a fluorescein isothiocyanate-conjugated monoclonal antibody (MAb; Dako, Glostrup, Denmark) directed to the NP.

^{51}Cr release assays. Target cells were resuspended in RPMI 1640 medium supplemented with 0.1% BSA and antibiotics (R0.1B). Next, 5×10^5 target cells were labeled with 50 μCi of $Na_2[^{51}Cr]O_4$ in R0.1B for 1 h at 37°C. After incubation, the cells were washed three times with R10F and adjusted to a concentration of 10^5/ml. Subsequently, 50-μl samples of target cells were incubated with PBMC stimulated in vitro with influenza virus A/NL/94-384G or A/NL/94-384R at effector cell/target cell (E/T) ratios of 80, 40, 20, 10, or 5 or with purified $CD8^+$-T-cell populations or T-cell clones at E/T ratios of 10, 5, 2.5, and 1. Target cells were also incubated with 100 μl of 10% Triton X-100 or R10F to determine maximum release and spontaneous release, respectively. After 4 h of incubation at 37°C, the supernatants were harvested (Skatron Instruments, Sterling, Va.), and radioactivity was measured by gamma counting. The percentage of specific lysis was calculated with the following formula: (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100%. ^{51}Cr release assays were performed in triplicate or quadruplicate, and the data are presented as averages.

Intracellular IFN-γ staining and flow cytometry. PBMC expanded in vitro after stimulation with influenza virus A/NL/94-384G or A/NL/94-384R were resuspended and adjusted to a concentration of 10^6 cells/ml in R10F supplemented with Golgistop (monensin; Pharmingen, Alphen a/d Rijn, The Netherlands). A total of 100,000 effector cells were incubated with 2×10^5 stimulator cells, which had been infected or incubated with rNP or peptides or left un-

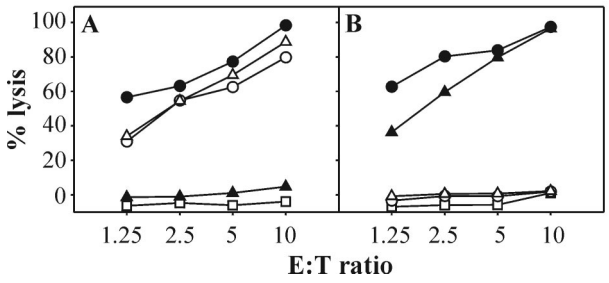

FIG. 1. Recognition of BLCL infected with recombinant influenza viruses by CTL clones. HLA-A*0201- and HLA-B*2705-positive BLCL were infected with influenza virus A/NL/94-384G (○) or A/NL/94-384R (●), pulsed with $M1_{58–66}$ (△) or $NP_{383–391}$ (▲) peptide or left untreated (□), and used as target cells for $CD8^+$-T-cell clones specific for the HLA-A*0201-restricted $M1_{58–66}$ epitope (A) and the HLA-B*2705-restricted $NP_{383–391}$ epitope (B) in a ^{51}Cr release assay. CTL clones were added at different E/T ratios as indicated, and specific lysis was calculated.

treated, for 6 h at 37°C in U-bottom plates. Subsequently, intracellular IFN-γ staining was performed as described previously (6). In brief, the cells were washed with PBS containing 2% FCS (P2F) and Golgistop, stained with MAbs directed to CD3 (Becton Dickinson, Alphen a/d Rijn, The Netherlands) and CD8 (Dako), fixed and permeabilized with Cytofix and Cytoperm (Pharmingen), and stained with an MAb specific for IFN-γ (Pharmingen). At least 10^3 gated $CD3^+$ $CD8^+$ events were acquired by using a FACSCalibur (Becton Dickinson) flow cytometer. The data were analyzed by using the software program Cell Quest Pro (Becton Dickinson). The data were expressed as the percentage of virus-specific IFN-γ-positive (IFN-γ^+) $CD8^+$ T cells in PBMC cultures and as the percentage of virus-specific IFN-γ^+ cells in the $CD8^+$-T-cell fraction. The relative reduction in the virus-specific IFN-γ^+ $CD8^+$ response was calculated according to the following formula: 100 − [(percentage of IFN-γ^+ $CD8^+$ T cells after restimulation with influenza virus A/NL/94-384G × 100)/percentage of IFN-γ^+ $CD8^+$ T cells after restimulation with influenza virus A/NL/94-384R)]. Routine staining with MAbs was carried out with 1×10^5 to 5×10^5 cells for 30 min at 4°C. In this way, the proportions of $CD3^+$, $CD4^+$, and $CD8^+$ cells could be determined.

Statistical analysis. It was assumed that the data obtained were not normally distributed because of the use of different blood donors. Therefore, statistical analysis was performed on the difference between A/NL/94-384G- and A/NL/94-384R-stimulated PBMC in the responses to influenza virus A/NL/94-384G- and A/NL/94-384R-infected target cells by using a one-way analysis of variance (ANOVA).

RESULTS

Validation of infection with influenza viruses A/NL/94-384G and A/NL/94-384R in vitro. To verify that target cells were equally susceptible to infection with influenza virus A/NL/94-384G and A/NL/94-384R, immunofluorescence assays were performed with BLCL infected with either virus. It was found that the numbers of infected cells were similar, as determined by positive staining for NP (data not shown). In addition, the expression of MHC class I-bound epitopes on the surface of BLCL infected with influenza A viruses A/NL/94-384G and A/NL/94-384R and the subsequent recognition by CTL were compared to confirm that they were on the same order of magnitude (Fig. 1). To this end, ^{51}Cr release assays were performed with HLA-A*0201- and HLA-B*2705-positive BLCL as target cells and $CD8^+$-T-cell clones specific for the conserved epitope from the matrix protein ($M1_{58–66}$, restricted by HLA-A*0201) and the HLA-B*2705-restricted $NP_{383–391}$ epitope. As expected, the CTL clone specific for the original $NP_{383–391}$ epitope recognized target cells infected with influenza virus A/NL/94-384R and failed to recognize those infected with influenza virus A/NL/94-384G (Fig. 1B). In contrast, the CTL clone specific for $M1_{58–66}$ recognized A/NL/94-384G- and A/NL/94-384R-infected target cells equally well, indicating that the infection of cells and the processing and presentation of immunogenic peptides were comparable for both viruses (Fig. 1A).

In vitro stimulation of PBMC with recombinant influenza viruses. Viruses A/NL/94-384G and A/NL/94-384R were used for the stimulation of PBMC obtained from an HLA-A*0201- and HLA-B*2705-positive blood donor to demonstrate that the R384G mutation resulted in the depletion of a CTL response against the $NP_{383–391}$ epitope. As shown in Fig.2A to H, stimulation with A/NL/94-384G or A/NL/94-384R resulted in similar numbers of IFN-γ^+ $CD8^+$ T cells specific for the conserved HLA-A*0201-restricted $M1_{58–66}$ epitope and the HLA-B*2705-restricted $NP_{174–184}$ epitope, as measured after restimulation with peptide-pulsed BLCL. In contrast, the response to $NP_{383–391}$ was virtually absent in PBMC cultures stimulated with influenza virus A/NL/94-384G but not in those stimulated with influenza virus A/NL/94-384R. In the PBMC of donor 2, 10.6% of the $CD8^+$ T cells were specific for the $NP_{383–391}$ epitope (Fig. 2H). Similar results were observed for the other donors. The absence of $CD8^+$ T cells specific for the $NP_{383–391}$ epitope, as measured by intracellular IFN-γ staining, coincided with the lack of capacity of the PBMC culture to lyse target cells pulsed with the $NP_{383–391}$ peptide but not those pulsed with the $M1_{58–66}$ peptide or the $NP_{174–184}$ peptide (Fig. 2, bottom panels).

Magnitude of the influenza virus-specific CTL response in vitro. The contribution of the $NP_{383–391}$ epitope to the influenza A virus-specific CTL response was determined by measuring the proportions of $CD3^+$ $CD8^+$ IFN-γ^+ cells in PBMC cultures stimulated with influenza virus A/NL/94-384G or A/NL/94-384R. IFN-γ expression was induced by restimulation with autologous BLCL or C1R-B27 cells infected with the respective influenza viruses or incubated with rNP or peptides. Figure 3 shows such an analysis for donor 2 after restimulation with autologous BLCL. A response was observed after restimulation with BLCL incubated with the $NP_{383–391}$ peptide or rNP-HK in PBMC cultures expanded after stimulation with influenza virus A/NL/94-384R. Such a response was not observed after restimulation with rNP-NL, which lacks the $NP_{383–391}$ epitope (Fig. 3, right). After primary stimulation with influenza virus A/NL/94-384G, no response was observed after restimulation with the $NP_{383–391}$ peptide or rNP (Fig. 3, left). In addition, no differences were observed for the influenza virus A/NL/94-384G-stimulated culture in the in vitro recall response induced by influenza viruses A/NL/94-384G and A/NL/94-384R. However, the CTL response against influenza virus A/NL/94-384G was significantly lower than the response to influenza virus A/NL/94-384R in PBMC cultures that were stimulated with influenza virus A/NL/94-384R.

These differences are depicted for all donors tested as the percentage of virus-specific IFN-γ^+ $CD8^+$ T cells in PBMC cultures (Fig. 4A) and as the percentage of virus-specific IFN-γ^+ cells in the $CD8^+$-T-cell fraction (Fig. 4B), ranging from 1 to 9% and 1 to 20%, respectively. Since no differences were observed after primary stimulation with a virus lacking the $NP_{383–391}$ epitope, these results show that depletion of this epitope resulted in a substantial decrease in the influenza A virus-specific CTL response in vitro. The relative reduction in the CTL response in-

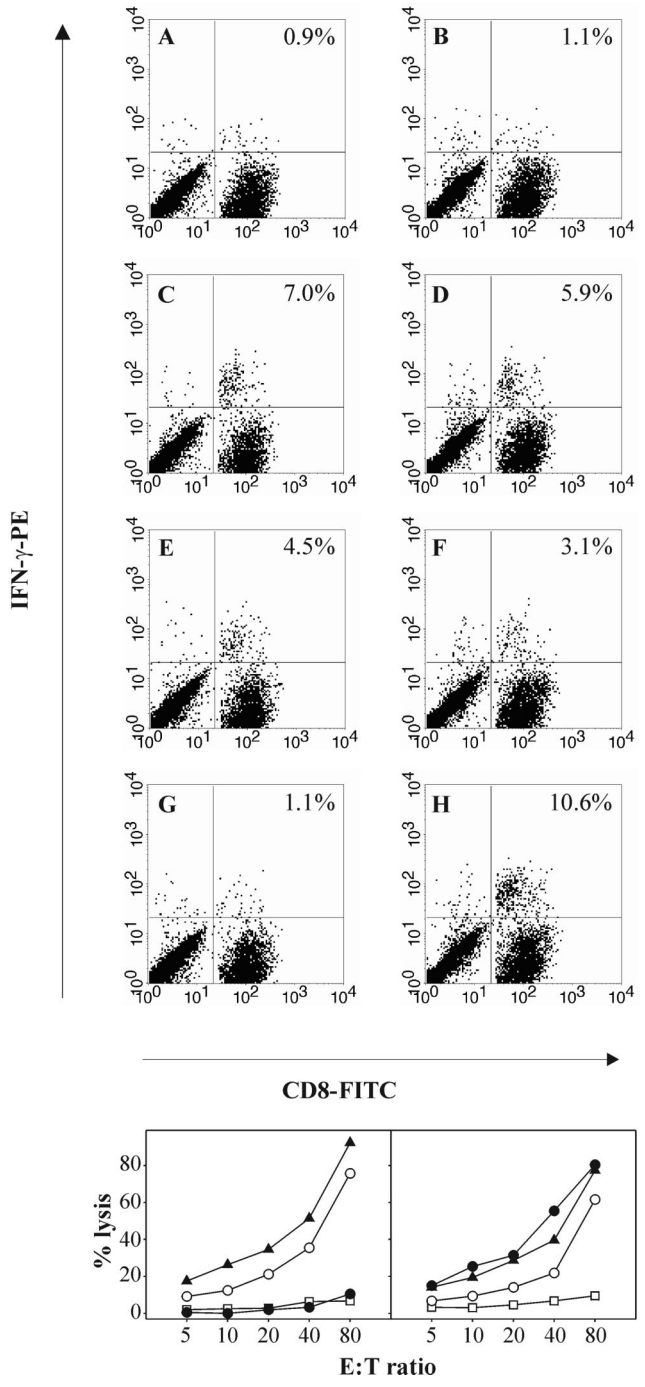

FIG. 2. IFN-γ expression and lysis by $CD3^+$ $CD8^+$ cells after stimulation of PBMC with influenza virus A/NL/94-384G or A/NL/94-384R. (A to H) PBMC from an HLA-A*0201- and HLA-B*2705-positive donor, expanded after stimulation with influenza virus A/NL/94-384G (A, C, E, and G) or A/NL/94-384R (B, D, F, and H), were restimulated with BLCL pulsed with the $M1_{58–66}$ epitope (C and D), the $NP_{174–184}$ epitope (E and F), or the $NP_{383–391}$ epitope (G and H). Restimulation with untreated cells was used as a negative control (A and B). Virus-specific CTL were visualized after staining with MAbs specific for CD3, CD8, and IFN-γ. The data represent the percentages of IFN-γ^+ cells (IFN-γ-PE) within the $CD8^+$-T-cell population. FITC, fluorescein isothiocyanate. (Bottom panels) CTL specific for the $M1_{58–66}$ epitope (▲), the $NP_{174–184}$ epitope (○), and the $NP_{383–391}$ epitope (●) were also detected by a ^{51}Cr release assay with peptide-pulsed BLCL as target cells and PBMC cultures stimulated with A/NL/94-384G (left) or A/NL/94-384R (right). Untreated cells were included as negative controls (□). Effector cells were added at different E/T ratios as indicated, and specific lysis was calculated.

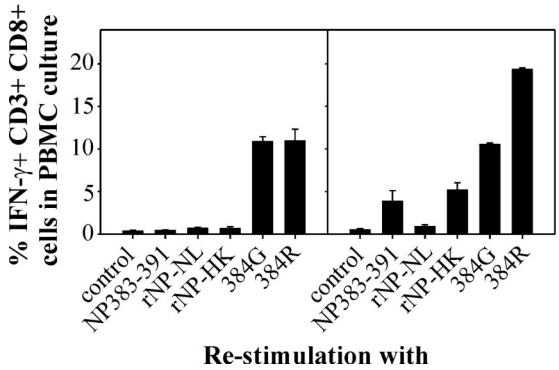

FIG. 3. Percentages of virus-specific $CD8^+$ T cells in PBMC cultures stimulated in vitro. The percentages of IFN-γ^+ $CD8^+$ T cells in PBMC from donor 2 were determined after stimulation in vitro with influenza virus A/NL/94-384G (left) or A/NL/94-384R (right), without or with the HLA-B*2705-restricted $NP_{383–391}$ epitope, respectively. Expanded cells were restimulated with autologous BLCL that were infected with influenza virus A/NL/94-384G or A/NL/94-384R or that were incubated with peptide $NP_{383–391}$ (SRYWAIRTR), rNP-HK, or rNP-NL. Virus-specific CTL were visualized after staining with MAbs specific for CD3, CD8, and IFN-γ. The data represent the proportions of $CD3^+$ $CD8^+$ IFN-γ^+ cells in total PBMC cultures. These values were calculated as the product of the percentage of IFN-γ^+ cells in the $CD3^+$ $CD8^+$ fraction multiplied by the percentage of $CD8^+$ T cells in the PBMC culture. Error bars indicate standard deviations.

duced by the virus without the $NP_{383–391}$ epitope compared to the response against the virus with the $NP_{383–391}$ epitope ranged from 2 to 46%, with a median of 30% (Fig. 4).

When C1R-B27 cells were used for the restimulation of PBMC cultures stimulated with influenza virus A/NL/94-384G or A/NL/94-384R, comparable results were obtained (data not shown). As expected, the percentage of virus-specific CTL restricted by a single HLA allele was lower than the percentage measured after restimulation with autologous BLCL. On average, the HLA-B*2705-restricted response constituted 65% of the response measured over all HLA alleles when autologous BLCL were used.

PBMC cultures stimulated with influenza viruses A/NL/94-384G and A/NL/94-384R were also tested in parallel in ^{51}Cr release assays. In contrast to the results obtained with intracellular IFN-γ staining, no clear differences were observed for the lysis of target cells infected with influenza virus A/NL/94-384G or A/NL/94-384R by PBMC expanded after stimulation with influenza virus A/NL/94-384R (Fig. 5A and B). However, when protein-labeled BLCL or C1R-B27 cells were used as target cells, PBMC cultures stimulated with influenza virus A/NL/94-384R recognized target cells labeled with rNP-HK but failed to recognize target cells labeled with rNP-NL, containing the R384G mutation (Fig. 5D). PBMC cultures stimulated with influenza virus A/NL/94-384G failed to recognize protein-labeled target cells at all (Fig. 5C). These findings indicate that the $NP_{383–391}$ epitope constitutes an important CTL epitope recognized in the NP-specific CTL response of these donors.

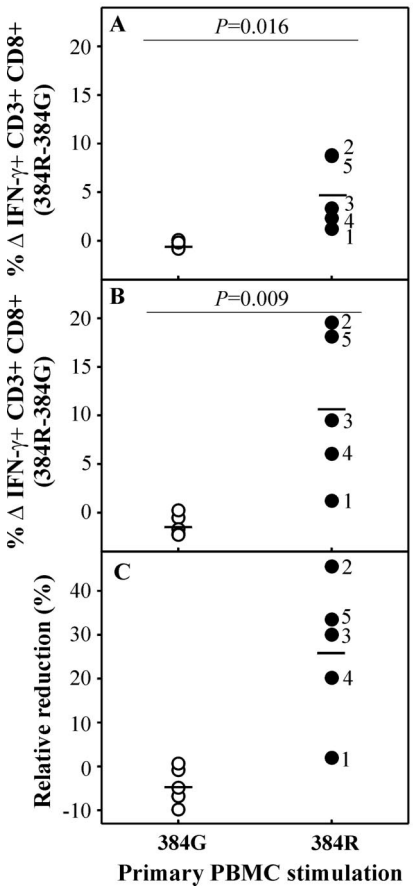

FIG. 4. Reduction of the in vitro CTL response through loss of the $NP_{383–391}$ epitope. (A) Reduction (Δ) in the percentages of virus-specific IFN-γ^+ $CD8^+$ T cells in PBMC cultures stimulated with influenza virus A/NL/94-384G or A/NL/94-384R for all five donors. Reduction was calculated by subtracting the percentage of IFN-γ^+ $CD8^+$ T cells after restimulation with influenza virus A/NL/94-384G from the percentage of IFN-γ^+ $CD8^+$ T cells after restimulation with influenza virus A/NL/94-384R. The percentage of IFN-γ^+ $CD8^+$ T cells in PBMC cultures was determined as indicated in the legend to Fig. 3. (B) Reduction expressed as the difference in the percentages of virus-specific IFN-γ^+ T cells within the $CD3^+$ $CD8^+$ fraction of PBMC cultures, as measured by flow cytometry. (C) Reduction in the percentages of virus-specific IFN-γ^+ $CD8^+$ T cells in PBMC cultures calculated as relative reduction as follows: 100 − [(percentage of IFN-γ^+ $CD8^+$ T cells after restimulation with influenza virus A/NL/94-384G × 100)/ percentage of IFN-γ^+ $CD8^+$ T cells after restimulation with influenza virus A/NL/94-384R]. In all three panels, the average is shown as a horizontal bar. Numbers refer to the donors listed in Table 2.

DISCUSSION

In the present study, the effect of a single amino acid substitution in epitope $NP_{383–391}$ on the in vitro human CTL response specific for influenza A virus was investigated by using recombinant influenza viruses differing only at position 384 of the NP. When these influenza viruses were used for the stimulation of PBMC obtained from HLA-B*2705-positive individuals, it was found that the mutation reduced the magnitude of the virus-specific CTL response in vitro.

Before influenza viruses A/NL/94-384G and A/NL/94-384R were compared for their capacities to induce CTL responses in vitro, it was confirmed that BLCL and C1R cells were equally susceptible to infection with these two viruses and that the antigen processing and presentation of MHC class I peptide complexes on the surface of these cells were on the same order of magnitude when a CTL clone specific for the $M1_{58–66}$ epitope was used. Furthermore, the presence and absence of the $NP_{383–391}$ epitope in influenza viruses A/NL/94-384R and A/NL/94-384G, respectively, were confirmed by using an $NP_{383–391}$-specific CTL clone and PBMC that were obtained from HLA-B*2705-positive individuals and that were stimulated in vitro with these viruses.

Stimulation of HLA-B*2705-positive PBMC with influenza virus A/NL/94-384G did not result in stronger responses against

TABLE 1. Known influenza A virus CTL epitopes likely recognized by the five individuals included in this study[a]

HLA restriction	Protein (amino acids)	Amino acid sequence
A*01	PB1 (591–599)	VSDGGPNLY
	NP (44–52)	CTELKLSDY
A*0201	M1 (58–66)	GILGFVFTL
	NS1 (122–130)	AIMDKNIIL
	NA (213–221)	CVNGSCFTV
	PA (46–54)	FMYSDFHFI
	PA (225–233)	SLENFRAYV
	PB1 (413–421)	NMLSTVLGV
	NA (75–84)	SLCPIRGWAI
A*03	NP (265–273)	ILRGSVAHK
	M1 (27–35)	RLEDVFAGK
A*1101	HA (63–70)	GIAPLQLGK
	HA (149–158)	VTAACSHAGK
	HA (450–460)	RTLDFHDSNVK
	M1 (13–21)	SIIPSGPLK
	NP (188–198)	TMVMELVRMIK
B*08	NP (380–388)	ELRSRYWAI
B*2705	NP (383–391)	SRYWAIRTR
	NP (174–184)	RRSGAAGAAVK

[a] Data are from the Influenza Sequence Database (http://www.flu.lanl.gov) (26).

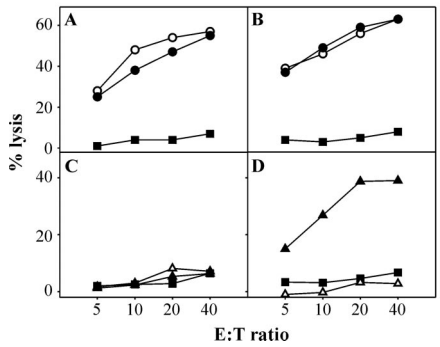

FIG. 5. Recognition of BLCL infected with influenza viruses (A and B) or incubated with rNP (C and D) by in vitro-stimulated PBMC obtained from donor 2. PBMC expanded after stimulation with influenza virus A/NL/94-384G (A and C) or A/NL/94-384R (B and D) were used as effector cells in ^{51}Cr release assays. Autologous BLCL infected with influenza virus A/NL/94-384G (○) or A/NL/94-384R (●) or BLCL incubated with rNP derived from influenza virus A/NL/18/94 (△) or A/HK/2/68 (▲) were used as target cells. Untreated cells were included as negative controls (■). The data represent the percentages of specific lysis at the indicated E/T ratios.

TABLE 2. HLA-A and HLA-B genotypes of the five individuals included in this study and proportions of virus-specific $CD8^+$ CTL and of CTL specific for $NP_{383–391}$ in PBMC stimulated with influenza virus A/NL/94-384R[a]

Donor	HLA-A and -B genotype	% of $CD8^+$ T cells specific for:		Relative proportion of $NP_{383–391}$
		A/NL/94-384R	$NP_{383–391}$	
1	A*0101 A*0201 B*0801 B*2705	62.0	4.6	7.4
2	A*0101 A*0201 B*0801 B*2705	42.9	10.0	23.3
3	A*0301 A*2301 B*2705 B*4101	31.6	8.6	27.2
4	A*1101 A*3101 B*2705 B*2705	29.9	5.0	16.7
5	A*0101 A*0201 B*0801 B*2705	42.5	8.6	20.2

[a] The percentage of $CD8^+$ T cells was determined by intracellular IFN-γ staining after in vitro restimulation with influenza virus A/NL/94-384R and after restimulation with $NP_{383–391}$ peptide-pulsed autologous BLCL. The relative proportion of $NP_{383–391}$-specific $CD8^+$ T cells was calculated with the following formula: (percent $NP_{383–391}$ specific/percent virus specific) × 100. The averages of two independently repeated experiments are given.

conserved epitopes $NP_{174–184}$ and $M1_{58–66}$ than did stimulation with influenza virus A/NL/94-384R, indicating that the loss of one HLA-B*2705-restricted epitope was not compensated for by the response to another in this in vitro system. In PBMC cultures of five HLA-B*2705-positive individuals, expanded after stimulation with influenza virus A/NL/94-384R, $CD8^+$ T cells specific for the $NP_{383–391}$ peptide and rNP-HK but not rNP-NL were demonstrated. The results obtained with rNP-HK or rNP-NL in intracellular IFN-γ staining and ^{51}Cr release assays indicated that the $NP_{383–391}$ epitope is the most immunodominant epitope in the NP restricted by HLA-B*2705 and other alleles expressed in the individuals tested (HLA-A*0101, HLA-A*0201, HLA-A*0301, HLA-A*1101, HLA-A*2301, HLA-A*3101, HLA-B*0801, and HLA-B*4101) (Tables 1 and 2). The responses to influenza virus A/NL/94-384G were similar in PBMC stimulated with influenza viruses A/NL/94-384G and A/NL/94-384R. However, in PBMC stimulated with influenza virus A/NL/94-384R, a lower frequency of influenza virus A/NL/94-384G-specific cells than of influenza virus A/NL/94-384R-specific cells was observed. This difference was used as a measure of the reduction in the virus-specific CTL response by the loss of the $NP_{383–391}$ epitope. In four out of five donors tested, a significant reduction in the number of influenza A virus-specific $CD8^+$ CTL was measured. Thus, the loss of a single epitope can have a major impact on the CTL response in vitro. The absence of a reduction in donor 1 correlated with the subdominant nature of the $NP_{383–391}$ epitope in this donor (Table 2). Overall, there was a correlation between the frequency of $NP_{383–391}$-specific CTL in PBMC cultures stimulated with A/NL/94-384R and the reduction in the CTL response after stimulation with an influenza virus lacking the $NP_{383–391}$ epitope.

Since three of the five donors tested were also HLA-B*0801 positive, it is possible that the loss of the HLA-B*0801-restricted $NP_{380–388}$ epitope also contributed to the reduction in the CTL response in these donors. It should be noted, however, that the $NP_{380–388}$ epitope is a minor epitope, especially in the presence of an HLA-B*2705-restricted response (4); therefore, its loss could be only marginally responsible for the observed reduction in the CTL response.

The differences in CTL responses measured by intracellular IFN-γ staining were not detected in ^{51}Cr release assays when virus-infected BLCL were used as target cells. The resolution of the ^{51}Cr release assays may not be sufficient to detect such differences, considering the reduction in frequency (1 to 9%) of virus-specific cells in the in vitro-expanded PBMC (which were used as effector cells in these assays) caused by the deletion of the $NP_{383–391}$ epitope.

Based on these data, we conclude that the mutation in the $NP_{383–391}$ epitope impaired the overall in vitro CTL response directed against influenza virus significantly. However, it is not clear from these studies what the impact of the R384G mutation on the in vivo CTL response in humans is. Of interest, it was recently demonstrated with a mouse model for influenza virus that deletion of a dominant *H-2D*^{b}-restricted epitope from the NP ($NP_{366–374}$) of influenza A viruses by site-directed mutagenesis resulted in the loss of a CTL response specific for this epitope in vivo. This finding correlated with a prolonged duration of viral shedding in infected mice and increased mortality rates (43). Furthermore, the loss of an immunodominant epitope was responsible for prolonged viral shedding (up to 2 months) in RAG-1-deficient mice transgenic for the T-cell receptor specific for that epitope (35). If infection of humans also results in reduced control of an infection with CTL epitope mutant influenza viruses, it might be expected that HLA-B*2705-positive individuals would be more susceptible to infection with a mutant virus (lacking the $NP_{383–391}$ epitope) than with a wild-type virus. Of interest, prolonged viral shedding in a small proportion of individuals in the human population (e.g., 8% HLA-B*2705 positive) was sufficient to explain the rapid fixation of the R384G substitution in the CTL $NP_{383–391}$ epitope at the population level with a recently developed theoretical model (16, 41).

Collectively, the data obtained in the present study showed that the loss of an immunodominant epitope through a mutation at an anchor residue affects the CTL response in vitro significantly. It could be speculated that the emergence of influenza A viruses with mutations in CTL epitopes have a profound advantage in individuals expressing the corresponding HLA molecules, eventually leading to the rapid fixation of these mutants (16).

ACKNOWLEDGMENTS

This work was supported by Novaflu-EU grant QLRT-2001-01034. R. A. M. Fouchier is a fellow of the Royal Dutch Academy of Arts and Sciences.

We thank W. Levering for performing the HLA typing and S. Sakko for organizing the collection of blood from blood donors (Sanquin Bloodbank). We also thank M. M. Geelhoed-Mieras for excellent technical assistance.

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