Viral Escape by Selection of Cytotoxic T Cell-resistant Variants in Influenza A Virus Pneumonia

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Abstract

Antigenic variation is a strategy exploited by influenza viruses to promote survival in the face of the host adaptive immune response and constitutes a major obstacle to efficient vaccine development. Thus, variation in the surface glycoproteins hemagglutinin and neuraminidase is reflected by changes in susceptibility to antibody neutralization. This has led to the current view that antibody-mediated selection of influenza A viruses constitutes the basis for annual influenza epidemics and periodic pandemics. However, infection with this virus elicits a vigorous protective CD8\(^+\) cytotoxic T lymphocyte (CTL) response, suggesting that CD8\(^+\) CTLs might exert selection pressure on the virus. Studies with influenza A virus-infected transgenic mice bearing a T cell receptor (TCR) specific for viral nucleoprotein reveal that virus reemergence and persistence occurs weeks after the acute infection has apparently been controlled. The persisting virus is no longer recognized by CTLs, indicating that amino acid changes in the major viral nucleoprotein CTL epitope can be rapidly accumulated in vivo. These mutations lead to a total or partial loss of recognition by polyclonal CTLs by affecting presentation of viral peptide by class I major histocompatibility complex (MHC) molecules, or by interfering with TCR recognition of the mutant peptide-MHC complex. These data illustrate the distinct features of pulmonary immunity in selection of CTL escape variants. The likelihood of emergence and the biological impact of CTL escape variants on the clinical outcome of influenza pneumonia in an immunocompetent host, which is relevant for the design of preventive vaccines against this and other respiratory viral infections, are discussed.

Key words: CD8\(^+\) CTL escape variants • viral persistence • influenza A virus • T cell receptor transgenic mice • influenza viral pneumonia

Introduction

In the adaptive immune response to most viruses, both T cells and neutralizing antibody play complementary roles in eliminating virus and promoting recovery (1, 2). During viral infections, the host aims to eliminate the virus and minimize associated pathology, whereas viruses have developed a remarkable variety of means to circumvent or suppress host responses to allow persistence and dissemination to new hosts over extended time periods (for reviews, see references 2–9). While many DNA viruses have acquired host genes that interfere with the immune response, for example pox and herpes viruses (8, 10, 11), RNA viruses cannot tolerate a large genome because of the low fidelity inherent to RNA replication, and as a consequence, rely on other means, such as rapid variation, to survive and persist. Thus, genetic variation is a major strategy exploited by viruses to evade host immune pressure and constitutes a major obstacle to efficient vaccine development. Variation occurs by several different mechanisms, including mutation, recombination, and reassortment for viruses with a segmented genome, and different virus families utilize these to different extents. As a result, RNA viruses exist as complex, dynamic mixtures of heterogeneous populations, termed quasispecies (12). If a virus is replicating under a constant set of environmental conditions, the consensus sequence of the viral population will remain unchanged. However, if conditions alter, for example under selective pressure of the host immune response, mutations that confer an increase in fitness (the relative ability of the virus to produce infectious progeny) will be selected for and come to predominate in the viral population.
Influenza A viruses have a single-stranded, segmented negative sense RNA genome characterized by its high degree of variability and the ability to cause acute respiratory infections of humans and animals, often resulting in significant morbidity and mortality (13, 14). A large body of experimental evidence suggests an essential role for neutralizing antibodies and CD8+ CTLs in eliminating influenza virus and promoting recovery of the host (1, 15–20). Although vigorous antibody responses directed against the surface glycoproteins hemagglutinin (HA) and, to a lesser extent, neuraminidase are generated against the virus, cross-protective humoral immunity against multiple virus strains does not occur due to the inherent antigenic variability of these proteins (21–24). This allows sequential re-infection of individuals over successive years by influenza viruses bearing antigenically distinct surface glycoproteins. Influenza virus variation involves antigenic “shift and drift.” Antigenic “shift” may be brought about by reassortment of viral genes, due to the segmented nature of the influenza virus genome, whereas antigenic “drift” occurs within HA and neuraminidase subtypes, and involves accumulation of minor genetic changes (usually point mutations; for reviews, see references 13, 25–27). Evidence for the role of humoral immunity in selection of variants of influenza virus surface proteins is overwhelming, with evolution of viral surface proteins occurring along a single lineage defined by accumulation of amino acid substitutions in neutralizing antibody target sites on HA, largely within the HA1 subdomain. In marked contrast to the subtype specificity of B cell, and thus antibody, recognition, human and murine MHC class I-restricted CD8+ CTL responses are usually cross-reactive between subtypes of influenza virus (15, 19, 28, 29). Consistent with this cross-reactivity is the relative conservation of the dominant CTL target antigens, usually internal viral proteins that are expressed early in infected cells, before progeny viruses release, and are inaccessible to antibody selection pressure.

The current consensus is that the acute nature of influenza A virus infection is unlikely to allow oscillations of CTL epitopes. However, the observations in this report, using mice transgenic for an influenza nucleoprotein (NP)366-374 peptide–specific TCR, demonstrate selection of CTL escape variants of influenza virus. Thus, CD8+ CTL responses directed to an internal viral protein can exert selective pressure on the virus, and variants containing point mutations in the NP366-374 epitope readily emerge. These mutations permit infected cells not only to escape transgenic CTL attack by interfering with TCR recognition of the mutant peptide–MHC complex, but surprisingly, also allow escape from polyclonal CTL recognition by affecting peptide binding to MHC class I. Such mutations facilitate viral reemergence and persistence, which occur weeks after the acute viral infection has apparently been resolved. The data demonstrate that amino acid substitutions in the major viral NP CTL epitope can be rapidly generated and selected in vivo. In addition, this highlights possible limitations of vaccination strategies based on enhancement and acceleration of virus-specific CD8+ CTL responses in influenza pneumonia.

Materials and Methods

Mice and Virus Infection. F5 transgenic mice expressing a TCR specific for influenza peptide NP366-374 (30, 31), F5 mice with a targeted mutation of recombination activating gene 1 (F5-RAG-1−/−) (32, 33), and RAG-1−/− mice were provided by Dr. D. Kiousis (National Institute of Medical Research, London, U.K.). Animals were bred and maintained in a pathogen-free environment. All procedures involving mice were conducted in strict accordance with institutional guidelines for animal care. For infection, animals were anesthetized with methoxyflurane (M etofane; Pitman-Moore) and inoculated intranasally with 50 μl of different influenza virus doses diluted in PBS containing 0.1% BSA.

Viruses. Influenza virus strain A/Memphis/102/72 (H3N2) was obtained from Dr. J.J. Skehel (National Institute of Medical Research, London, U.K.). Viruses were propagated in 10圣经-embryonated hen eggs, and viruses were determined by plaque assay using Madin-Darby canine kidney (MDCK) cells as described previously (34). Isolates of influenza A virus derived from lungs of infected mice were cloned by three rounds of plaque purification and propagated on MDCK cells.

Viruses Titered in Lung Tissue. Tissues from infected mice were homogenized in 1 ml of cold PBS, and 50 μl of log dilutions of clarified homogenates were adsorbed for 1 h at 37°C onto confluent monolayers of MDCK cells in 96-well plates. Infected monolayers were then overlaid with MEM supplemented with 0.5% BSA and tosylamido-phenylethyl-chloromethyl ketone (TPCK)-trypsin (250 μg/ml; Sigma-Aldrich) and incubated for 72 h at 37°C and 5% CO2. Virus growth was assessed by HA assay with 1% chicken erythrocytes. 50% tissue culture infectious dose (TCID50) was determined by the method of moving averages (35), and virus titers are expressed as TCID50/g tissue. The threshold of virus detection in the MDCK assay is ~102 TCID50/g lung tissue.

Viral Peptides. Peptides were synthesized at The Medical College of Georgia Molecular Biology Core Facility, using a Perkin-Elmer 433A peptide synthesizer. The peptides used in this study were the H-2Db-binding viral peptide NP366-374 (ASNENMDT M) of A/Memphis/102/72 or its CTL escape variants NP370 (ASNEMDT M), NP370 (ASNEMDT M), NP370 (ASNEMDT M), NP370 (ASNEMDT M), or the H-2Kb-binding influenza virus peptide nonstructural protein 2 (NS2)114-121 (RTFSFDGL) (36).

Cytotoxicity Assay. Cell-mediated cytotoxicity was determined in a standard 5-hour microcytotoxicity test as described (32, 37). EL-4 (H-2b) cells infected with virus or loaded with peptide were used as target cells. Cells infected with virus-infected target cells EL-4 cells were washed in serum-free medium, and virus at a multiplicity of infection of 5 was added at the time of 51Cr labeling. Effector cells were in vitro activated for 18 h with NP366-374 or NS2114-121 peptide-pulsed (0.1 μg/ml) irradiated (30 Gy) splenocytes (4 x 109) in 2 ml of IMDM (GIBCO BRL) supplemented with 10% FCS and...
cytes loaded with the corresponding peptide. D-gated rat mAb specific to mouse CD8, respectively) were used to identify cytokine-positive cells. 

Staining of H-2D^b complexes on RMA-S cells by Synthetic Peptides. For stabilization assay, RMA-S cells were grown at 25°C (5% CO_2) for a minimum of 24 h to induce stable H-2D^b expression at the cell surface (38–40). 10^6 cells were mixed with varying concentrations of peptide in 100 μl IMDM medium containing 10% FCS, and then incubated in microtiter plates for 30 min at 25°C, followed by 4 h at 37°C. Cell surface expression of H-2D^b was determined using the H-2D^b-specific mouse mAb B22-249 (provided by Dr. U. Hämmerling, Sloan-Kettering Cancer Center, New York, NY), and FITC-conjugated goat anti-mouse IgG secondary antibody (Jackson Immunoresearch Laboratories). Cells were analyzed with a FACSCalibur™ flow cytometer (Becton Dickinson). 

Results

Reemergence and Persistence of Influenza A Virus in F5-R AG-1^-/^- Mice. The CD8^+ CTL response in F5-R AG-1^-/^- mice infected with 10^6 PFU of A/M emphis/102/72 promotes rapid reduction of pulmonary virus to below the limit of detection by TCID_50, by days 6–8 after infection, and the mice initially recover from primary viral pneumonia. However, if mice are left for a sufficient period (~60 d), they eventually sicken and die. This late mortality is associated with virus reemergence by days 20–24 and viral persistence in the lung for several weeks (Fig. 1). As virus control in F5-R AG-1^-/^- mice is meditated solely via CD8^+ CTLs arriving in the infected respiratory tract 2–3 d after peak lung viral titers (Fig. 1), this novel finding raised the possibility that A/M emphis/102/72 variants escaping immune recognition by transgenic CTLs had been selected, as initially shown for lymphocytic choriomeningitis virus (45).

Level of Virus Inoculum Influences the Range of Influenza Virus Variants. To verify that virus reemergence was due to lack of recognition by transgenic CTLs, virus recovered from lungs of A/M emphis/102/72-infected (10^6 PFU) F5-R AG-1^-/^- mice on days 4, 6, 20, 34, and 50 after infection was analyzed directly in a 51Cr-release assay with the fol-
lowing results: EL-4 (H-2b) target cells infected with bulk virus isolated on days 4 and 6 after infection, but not on days 22, 34, or 50, were sensitive to lysis by in vitro-activated transgenic CTLs at comparable levels to target cells infected with wild-type virus. In contrast, the same target cells were lysed efficiently by polyclonal CTLs specific to NP366–374 or by CTLs specific to the N52114–121 epitopes of A/Memphis/102/72 used as a control to verify appropriate infection of target cells in the assays (Fig. 2). These results were confirmed with plaque-purified isolates from mice infected 22 and 34 d previously (data not shown).

To ascertain that loss of CTL recognition was associated with viral genotype changes, we derived sequences corresponding to a region within the A/Memphis/102/72 NP gene (nucleotides 990–1307) comprising the NP366–374 CTL epitope. Virus recovered from lungs of infected mice (three per group) on days 4 and 6 did not show any nucleotide difference in the analyzed NP gene fragment. However, virus resistant to CTL lysis recovered from mice killed on days 22 and 34 after infection revealed identical results, indicating predominance of one virus variant type, containing an amino acid change (371M → I) at position 6 (the transgenic TCR recognition site) within the NP366–374 epitope. Sequencing plaque-purified isolates (five per virus sample) gave identical results (data not shown), confirming rapid in vivo selection of virus variants resistant to lysis by transgenic CTLs but still recognized by polyclonal NP366–374-specific CTLs. Note that in vitro-restimulated polyclonal NP-specific CTLs from C57BL/6 mice contain a mixture of individual clonotypes, at least some of which will be cross-reactive between wild-type and peptide-bearing alterations at TCR contact residues (as shown in Fig. 2).

Next, the influence of virus inoculum on the frequency and spectrum of variant type in the reemergent virus was studied. Infection of transgenic mice with increasing doses of A/Memphis/102/72 (10³–10⁶ PFU) resulted in higher virus titers on days 2–4 and accelerated kinetics of virus reemergence in the lung. Thus, emergent virus was detected earlier in mice infected with 10⁴ PFU (day 18) than with 10² PFU (day 22). Analysis of lung virus isolates derived from mice infected 20 or 34 d previously revealed a pure population of CTL escape variants. Approximately half (15 of 35 total) of virus isolates recovered from mice after infection with relatively high doses (10⁴ to 10⁶ PFU) of A/Memphis/102/72 either sensitized target cell lysis by polyclonal NP366–374 peptide-specific CTLs to a lesser degree than wild-type A/Memphis/102/72, or were unable to sensitize target cell lysis at all (data not shown). The remainder of the isolates displayed the phenotype of the variants found in mice infected with 10² PFU, as they were unable to sensitize target cell lysis by transgenic cells but were still recognized by polyclonal CTLs.
Sequence analysis of these isolates revealed two patterns of mutation within the NP366–374 CTL epitope. The 15 isolates that resisted lysis by polyclonal CTLs displayed alterations to dominant anchor residues at position 5 or 9 within the NP366–374 CTL epitope (370N →S, 370N →D, 370N →T, or 374M →T). An equal number (15 of 35 total isolates) that sensitized target cells to lysis by polyclonal but not by transgenic CTLs displayed a single mutation at residue 6 or 7 (371M →I, 371M →V, 371M →T, or 372D →E) that comes into direct contact with the TCR. The remaining five isolates contained a mixture of two virus populations, with mutations 370N → S or 370N → T coexisting with 371M → I. Isolates containing double mutations were not obtained. This propensity for mutation was specific for the sequence of CTL epitope NP366–374, as no other changes were observed in the ~150 nucleotides flanking the epitope on each side. These results are summarized in Table I.

Presentation and CTL Recognition of the NP366–374 Epitope of Influenza Escape Variants. For further analysis, triple plaque-purified isolates were obtained for each of the variant types with a substitution in the NP366–374 peptide anchor residue at position 5 or 9 (designated A/M em/N P370S, A/M em/N P370D, A/M em/N P370T, or A/M em/N P374T), and for the main variant type with a substitution in the TCR contact site at position 6 (A/M em/N P371I). To determine whether the changes within the NP366–374 epitope affect presentation of the peptide by MHC molecules or affect recognition by specific CTLs, we synthesized peptides corresponding to the NP366–374 variant epitope sequences and tested them for their ability to bind H-2Db molecules (RMA-S H-2Db stabilization assay and soluble MHC- peptide binding assay). In addition, virus isolates and the corresponding mutant peptides were assayed for their ability to sensitize cells for lysis by in vitro-activated transgenic or polyclonal NP366–374-specific CTLs.

RMA-S cells lack a functional transporter associated with antigen processing (TAP)-2 gene, and express empty H-2Db molecules that can be stabilized by adding exogenous peptide (39). The ability of a peptide to stabilize the MHC molecule on the cell surface correlates with its affinity for the molecule. The results in Fig. 3 show that the NP366–374 peptide of A/M em/N P371I and wild-type viruses were comparable in stabilizing the H-2Db molecule. In repeated assays, the peptide of A/M em/N P374T was found to stabilize H-2Db expression slightly less well than for wild-type peptide (~10-fold more variant peptide is required for half-maximal stabilization than that of wild-type peptide). In contrast, the mutations in the NP366–374 peptide of A/

### Table I. CTL Escape Variants Displaying Point Mutations in the NP366–374 Epitope of A/M em/is/102/72 Influenza Virus

| Virus isolate | Nucleotide and deduced amino acid sequences | Number of variant isolates/total tested |
|---------------|------------------------------------------|--------------------------------------|
| A/M em/is/102/72 | GCT TCA AAT GAA AAC ATG GAT ACT ATG | 0/35 |
| A/M em/N P370S | ... ... ... ... | 3/35 |
| A/M em/N P370D | ... ... ... ... | 2/35 |
| A/M em/N P370T | ... ... ... ... | 8/35 |
| A/M em/N P374T | ... ... ... ... | 2/35 |
| A/M em/N P371I | ... ... ... ... | 18/35 (8/8)* |
| A/M em/N P371V | ... ... ... ... | 1/35 |
| A/M em/N P371T | ... ... ... ... | 1/35 |
| A/M em/N P372E | ... ... ... ... | 2/35 |

The nucleotide and deduced amino acid sequence of the NP366–374 epitope of wild-type and reemergent escape variants recovered from the lungs of F5-RAG-1/2/2 mice that were infected with relatively high dose (10^4–10^6 PFU) of A/M em/is/102/72 are indicated. The nucleotide sequence is given in viral sense, and differences in nucleotide or amino acid sequence are indicated in bold. The fraction of virus isolates displaying mutation within the NP366–374 CTL epitope was compared with the number of analyzed isolates.

*In addition, the pattern of mutation of isolates recovered from lungs of mice that were infected with a relatively low dose of virus (10^2 PFU) was analyzed, revealing one type of mutation, and is indicated in parenthesis. To avoid artifact, four DNAs derived from two independent PCR amplifications, utilizing the high fidelity Pfu polymerase, were sequenced for each virus variant, with the same results.
of H-2Db at the highest concentration assayed compared that the variant peptides stabilize a significantly lower level of peptide than wild-type was required. It should be noted that the variant peptides stabilize a significantly lower level of H-2Db molecule at 37°C was determined in dose-response experiments. Each peptide was analyzed in four independent experiments with similar results. Data are shown as H-2Db stabilization indices calculated as mean fluorescence increase in relation to the expression level of H-2Db in the absence of peptide, as indicated by the dashed line.

Figure 3. Relative binding of variant peptides to H-2Db molecules on the surface of RMA-S cells. The ability of the NP366-374 peptide of A/Memphis/102/72 (●) or its variants NP370S (▲), NP370D (▼), NP370T (■), NP374T (▲), or NP371I (○) to stabilize cell surface expression of H-2Db molecule at 37°C was determined in dose-response experiments. Each peptide was analyzed in four independent experiments with similar results. Data are shown as H-2Db stabilization indices calculated as mean fluorescence increase in relation to the expression level of H-2Db in the absence of peptide, as indicated by the dashed line.

Figure 4. Ability of the A/Memphis/102/72 variants to sensitize target cells for lysis by in vitro-stimulated transgenic or by polyclonal NP366-374 peptide-specific CTLs. Susceptibility of EL-4 (H-2b) target cells infected with the A/Memphis/102/72 or its variant viruses to lysis by transgenic CTLs (▲) or polyclonal CTLs specific to NP366-374 (●) or specific to NS2114-121 peptide (■) was evaluated in a 4:5-h standard cytotoxicity assay. Lysis of untreated target cells was <5% at the highest E/T cell ratio. Results are representative of several experiments.

M em/NP370S, A/M em/NP370D, or A/M em/NP370T have a major effect on the ability of the peptide to stabilize H-2Db at the surface of RMA-S cells. Thus, for half-maximal H-2Db stabilization, ~100-fold (370N→S), ~500-fold (370N→D), or ~10,000-fold (370N→T) more variant peptide than wild-type was required. It should be noted that the variant peptides stabilize a significantly lower level of H-2Db at the highest concentration assayed compared with the wild-type peptide. As an independent assay for binding affinity, the concentration of the peptide yielding 50% inhibition (IC50) of the binding of the radiolabeled adenovirus E1A analogue peptide (SGPSNTYPEI) to soluble H-2Db was measured as described by Vitiello et al. (36). The data confirmed the general peptide binding pattern found in the RMA-S stabilization assay. Thus, wild-type peptide bound with high affinity (IC50 6 nM), whereas the corresponding values for variant peptide were: 77 nM (374M→T), 118 nM (370N→S), 835 nM (370N→D), and 6,424 nM (370N→T). Variant peptide with change in the TCR contact site (371M→I) bound very efficiently to H-2Db (IC50 2 nM). Therefore, changes in the NP366-374 peptides of A/M em/NP370D, A/M em/NP370S, and A/M em/NP370T affect binding of the peptide to H-2Db molecules, whereas the change in A/M em/NP371I interferes with recognition by T effector cells. The alteration 374M→T moderately reduces the binding ability of the peptide to H-2Db in the above assays.

EL-4 target cells infected with A/M em/NS2114-121 showed a slightly reduced ability (~10-fold) to induce proliferation of transgenic CTLs, whereas a drastically reduced proliferative capacity was noticed with peptides of A/M em/NP370D (~250-fold), A/M em/NP370S (~500-fold), or A/M em/NP370T (~5,000-fold). No proliferation with the peptide of A/M em/NP374T was detected at the highest concentration tested (10 μg/ml). The approximate concentrations required for half-maximal proliferation are: wild-type peptide, 0.3 nM; variant peptides 374M→T, 2 nM; 370N→S 70 nM; 370N→D, 150 nM; and 370N→T, 2 μM. In contrast, transgenic cells showed significant proliferative responses only when stimulated with A/M em/NS2114-121-infected splenocytes (Fig. 5 B). This suggests that an insufficient MHC-peptide ligand density for proliferative responses can be produced.
during infection, but that a response can be triggered if enough peptide is given to presenting cells exogenously.

To test the efficiency of the variant NP<sub>366-374</sub> peptides to sensitize target cells for lysis, splenocytes from F5-RAG-1<sup>−/−</sup> mice were stimulated with the NP<sub>366-374</sub> peptide of wild-type virus and the specific CTLs generated were tested. As shown in Fig. 5 C, the variant peptides 370N→S, 370N→D, 370N→T, or 374M→T used at a final concentration of 10 μg/ml sensitize EL-4 target cells for lysis by transgenic CTLs as well as wild-type peptide. However, marked differences in dose-response were observed. In general agreement with the proliferation studies as detailed above, peptide with the mutation 374M→T sensitizes cells for lysis slightly less efficiently (<10-fold) than wild-type peptide, whereas at least 1,000-fold more variant peptide 370N→S or 370N→D, and 1,000,000-fold more 374N→T were required to sensitize cell lysis. Approximate concentrations required for half-maximal lysis: wild-type peptide, 1 pM; variant peptides 374N→T, 10 pM; 370N→S or 370N→D, 1 nM; and 370N→T, 1-5 μM. Variant peptide with mutation 371M→I did not sensitize cell lysis at all. In addition, F5-cells efficiently lysed wild-type virus-infected target cells, whereas no lysis was observed with the variant viruses (Fig. 5 D).

Behavior of CTL escape variant viruses in transgenic mice. The ability of variant peptides to induce proliferative responses and sensitize target cell lysis, which occurs at large peptide excess (>0.01 μg/ml) for peptides with mutation 370N→S, 370N→D, 370N→T, or in the case of 374M→T, slightly less well than wild-type peptide, raises the question of whether infection with the cognate viruses could trigger a CTL response in vivo. Thus, the capacity of variant viruses to escape recognition by transgenic or poly-clonal CD8<sup>+</sup> CTLs specific for the NP<sub>366-374</sub> epitope in vivo was evaluated. In contrast to A/M emph/102/72, the same dose of variants (A/M em/ NP<sub>370S</sub>, A/M em/ NP<sub>370D</sub>, A/M em/ NP<sub>370T</sub>, A/M em/ NP<sub>374T</sub>) persist in the lung at comparably high titers in F5-RAG-1<sup>−/−</sup> mice over the time period examined (Fig. 6, top), indicating that variant viruses did not trigger an antiviral CTL response.

Figure 5. Responses of F5-RAG-1<sup>−/−</sup> transgenic cells to A/M emph/102/72 variant viruses and corresponding NP<sub>366-374</sub> peptides. (A) T cell proliferative responses to peptide. F5-RAG-1<sup>−/−</sup> splen cells (10<sup>5</sup>/well) were stimulated with the given concentration of the appropriate peptide for 3 d. Proliferation was determined by incorporation of [3H]thymidine pulsed during the last 6 h of culture. Stimulation indices were calculated in relation to proliferation in medium control, for NP<sub>366-374</sub> peptide of A/M emph/102/72 (●), or its variants A/M em/ NP<sub>370S</sub> (▲), A/M em/ NP<sub>370D</sub> (▼), A/M em/ NP<sub>374T</sub> (▲), or A/M em/ NP<sub>371I</sub> (●). (B) Proliferative response to virus. Spleen cells from F5-RAG-1<sup>−/−</sup> mice (5×10<sup>5</sup>/well) were cultured with virally infected (30 Gy) splenocytes (5×10<sup>5</sup>/well) from C57BL/10 mice for 96 h. T cell proliferation was determined as described above. Bars A–F show proliferation of T cells with APCs infected with A/M emph/102/72, A/M em/ NP<sub>370S</sub>, A/M em/ NP<sub>370D</sub>, A/M em/ NP<sub>371I</sub> or A/M em/ NP<sub>374T</sub>, respectively. (C) Susceptibility of EL-4 target cells pulsed with given concentrations of peptide to lysis by transgenic cells. EL-4 cells labeled with 51Cr were added to 96-well plates containing the given concentration of peptides and incubated with F5-RAG-1<sup>−/−</sup> splenic activated in vitro with 0.1 μg/ml of NP<sub>366-374</sub> peptide. (D) Ability of variant viruses to sensitize target cells for lysis by transgenic cells. EL-4 cells labeled with 51Cr were added to 96-well plates containing the given concentration of peptides and incubated with F5-RAG-1<sup>−/−</sup> splenic activated in vitro with 0.1 μg/ml of NP<sub>366-374</sub> peptide of A/M emph/102/72 for 4-5 h. E/T cell ratio was 30:1 for all groups. Different peptides are indicated as in A. (D) Ability of variant viruses to sensitize target cell lysis by in vitro-activated transgenic T cells. This experiment is performed as described under C using EL-4 cells infected with the variants as target cells. Bars A–F represent target cells infected with the wild-type or variant virus as indicated in B.

Figure 6. Replication of A/M emph/102/72 or its variant viruses in the lung of F5-RAG-1<sup>−/−</sup> or RAG-1<sup>−/−</sup> mice. F5-RAG-1<sup>−/−</sup> (top) or B10-RAG-1<sup>−/−</sup> (bottom) mice were infected with 10<sup>6</sup> PFU of the indicated viruses. Virus titers, expressed as mean log<sub>10</sub> TCID<sub>50</sub> per gram of lung, were determined at the times indicated. Data points are expressed as mean log<sub>10</sub> ± SEM of three to five mice.
response in these mice. Similarly, all strains persisted at equally high levels in control RAG-1−/− mice, indicating that mutations in the NP366-374 epitope confer no disadvantage for viral replication in vivo (Fig. 6, bottom).

As engagement of TCRs by MHC-peptide complexes initiates subsequent signal transduction events resulting in cytotoxicity, cytokine secretion, and cell proliferation (46), it is possible, as has been shown for altered peptide ligands, that the variant viral peptides elicit some of the physiological T cell responses associated with full antigen stimulation while blocking the completion of others. Thus, it is possible in vivo that the variant N P366-374 peptides could induce clonal expansion and maturation without triggering cytolytic activity. To test this possibility, the recruitment and functional state of transgenic T cells recovered by BAL from F5-RAG-1−/− mice infected with 10⁴ PFU of A/Memphis/102/72, or its variants 9 d previously was studied. A/Memphis/102/72 infection triggered a rapid accumulation of large numbers of activated effector transgenic CTLs (CD44high and CD62Llow) in the lung. These CTLs efficiently lysed target cells sensitized with the wild-type NP366-374 peptide. In contrast, no transgenic T cells were detectable in the BAL of mice infected with A/Mem/ NP370S, A/Mem/N P370D, A/Mem/N P370T, A/Mem/N P374T, or A/Mem/N P371I viruses (Table II). Comparable results were obtained when BAL cells obtained from mice infected 16 d previously were tested (data not shown).

Table II. Inflammatory Response in the BAL of F5-RAG-1−/− Mice Infected with CTL Escape Variants of A/Memphis/102/72 Influenza A Virus

| Virus isolate  | Total BAL cells/mouse | F5 Tg cells/lung | Percentage of ex vivo CTL activity at F5 Tg E/T ratio: |
|---------------|-----------------------|------------------|--------------------------------------------------------|
|               | ×10⁶                  | ×10⁶             | 50 16 6 2                                               |
| A/Memphis/102/72 | 10.6 ± 1.4           | 9.4 ± 3.6        | 62 43 22 6                                             |
| A/Mem/NP370S   | 8.9 ± 3.8            | <0.1             | <5 - - -                                               |
| A/Mem/NP370D   | 22.4 ± 9.3           | <0.1             | <5 - - -                                               |
| A/Mem/NP370T   | 2.1 ± 0.8            | <0.1             | <5 - - -                                               |
| A/Mem/NP374T   | 5.3 ± 1.6            | <0.1             | <5 - - -                                               |
| A/Mem/NP371I   | 5.6 ± 2.4            | <0.1             | <5 - - -                                               |

The numbers of inflammatory cells in BAL are indicated as mean log₁₀ ± SEM per lung of three mice. Transgenic CTLs were detected in the same samples by staining cells with antibodies specific for Vβ11 and CD8 and analyzed by flow cytometry. Absolute numbers of transgenic CTLs were calculated as percentage of transgenic positive cells by flow cytometry multiplied by total cell number. Populations <0.1% were considered nondetectable. In the same experiment, the CTL activity was evaluated in a 4-5-h standard cytotoxicity assay. Lysis of uninfected target cells was <5% at the highest E/T ratio. Similar results were obtained in an additional experiment (data not shown). Tg, transgenic.
peptide of wild-type or variant virus (A/Mem/ NP370S, A/Mem/ NP370D, A/Mem/ NP370T, or A/Mem/ NP374T) upon stimulation in vitro. The CTL activity observed upon in vitro restimulation of splenocytes from mice after primary or secondary virus challenge correlates with the data from tetramer staining or intracellular staining of IFN-γ expression, indicating that mutations at position 5 or 9 of the NP366–374 epitope result in complete loss of recognition by CD8+ CTLs specific for that epitope (Fig. 8).

As epitope variants with mutations on residues of TCR contact sites can function as agonists or antagonists of antigen-specific T cells, we sought to examine whether infection of mice with A/Mem/NP371I (main variant type found in the population of reemergent virus) would impact the polyclonal CTL response specific to NP366–374 peptide. Infection with A/Mem/NP371I induced CD8+ T cells cross-reactive between wild-type and variant NP366–374 peptides, as detected with tetramers containing the NP366–374 of A/Mem/102/72 or by measuring CTL activity toward both wild-type or variant NP366–374 peptide in cultures of splenocytes during primary or recall infection (Figs. 7 and 8, and data not shown). This observation is in agreement with previous reports that variation on residues in contact with TCRs can induce cross-reactive CTLs (47, 48). However, a more detailed quantitative analysis of the CTL response in the BAL demonstrated that the number of cross-reactive CTLs, which comprise a small fraction of the CTLs in wild-type infected mice, was dramatically reduced in A/Mem/NP371I-infected mice. To better understand the ontogeny of the CTL population in these mice, we measured the ability of CD8+ T cells to initiate IFN-γ synthesis upon stimulation with the original (wild-type) or variant peptide. Intracellular IFN-γ staining of BAL cells revealed induction of a T cell subpopulation cross-reactive between the wild-type and variant peptide, as well as a population specific for the variant peptide alone (data not shown). In several experiments, we consistently found that the total magnitude of the NP366–374 peptide-specific CTL population differs greatly between wild-type and A/Mem/NP371I infection (3-10-fold more specific CD8+ T cells were detected in mice infected with wild-type versus variant). This suggests that exposure of a host to variants with substitutions on the TCR-exposed side can impair the CTL response to the equivalent epitope. From these experiments, we conclude that mutations in the NP366–374 epitope result in complete (mutation at anchor residue po-
Discussion

Antigenic variation affecting recognition by antibody or cytotoxic T cells is a strategy that viruses exploit to promote their survival in the face of the host adaptive immune system (7, 38, 49, 50). Although virus variants that are not recognized by epitope-specific CTLs have been shown to arise during several viral infections, in many cases their significance remains controversial. It has been argued that the immune response is plastic enough to contain their replication, and that escape at one epitope is unlikely to give material advantages to viral persistence because several CD8+ T cell epitopes may be recognized simultaneously by CTLs in an immunocompetent host. However, data derived from more recent studies in several viral systems strongly suggest that CTL escape plays a role in viral evolution, reinforcing the concept that CTL escape variants generated during infection may promote virus survival in the host (6, 7, 38, 49, 50). In line with this view, changes in CTL epitopes occurring as a result of immune pressure would generate antigenic variants of biological significance when the ability of the host to control viral infection is tenuous and their emergence may tip the balance to the pathogen, resulting in chronic infection. The lack of reported CTL escape variant selection in influenza has been ascribed to the acute nature of the infection and its confinement to the respiratory tract. In this regard, viruses that are known to be selected to some degree by CD8+ CTLs are thought to differ regarding pathogenesis, mechanisms of persistence, and mutation rates. However, the studies reported here suggest that the respiratory tract can act as a suitable site for generation of CTL escape variants of influenza virus, albeit under strong and highly directed selective pressure against an immunodominant epitope.

Here, transgenic CTLs in the absence of a virus-specific antibody response are sufficient to reduce the viral load in the lung below the threshold of detection by days 8–10 and virus remains undetectable for a period of 2–3 wk. However, at the end of this period, virus that is resistant to CTL recognition reemerges and persists at high titer in the lungs for several weeks until the mice succumb to viral infection. The time of viral reemergence and the repertoire of variant populations depend on the initial dose of infection. Thus, by increasing the dose of infection, virus reappears in the lung earlier and a more diverse mutant population is observed.

In general, the selection of CTL escape variants depends on the strength of CTL pressure, the rate of viral turnover, and the escape mutant fitness. In our model, the lung represents an ideal site for selection of CTL escape variants because the virus has several days to become established in the respiratory tract before CTLs appear at sites of virus replication, producing a pool of virus that is a potential source of variants. Influenza virus replication is substantially restricted in vivo, as generation of infectious virus by HA cleavage depends on a trypsin-like enzyme restricted to the respiratory tract (51, 52). As a consequence, the virus-specific CTL response is targeted to the respiratory tract and effector CTLs can reach extremely high frequencies in the BAL. The selection process in a respiratory viral infection profoundly differs from systemic infections where virus replication occurs at the same site as the induction of CTL responses. Thus, CTL-mediated pressure on the virus, in combination with other factors, is sufficient to lead to selection of escape variants. Two hypotheses can be envisaged: either CTL escape mutants were present in the initial viral inoculum and then selected, or they were generated during the ongoing infection and then selected under CTL pressure. Our data favor the second hypothesis, as bulk virus isolated on days 4 and 6 after infection was recognized efficiently by peptide-specific CTLs, and because we did not observe the rapid emergence of CTL escape...
variants that would be expected from outgrowth of preexisting variant population after CTL pressure. However, it remains to be determined precisely when CTL escape mutations first arise in infected transgenic mice.

A vital question concerns the site of persistence of the virus before reemergence within the lung. The viruses used in this study are pneumotropic and not reported to be capable of replication outside the respiratory tract. Furthermore, the variants retain the ability to induce cytopathic effect when grown in vitro. This suggests that rather than true latency, virus replication is sustained by transmission from cell to cell within the respiratory tract, albeit at levels below the limit of detection by TCID₅₀, until a point is reached where inflammation and tissue damage induced by the acute infection in the lung is resolved to a sufficient extent to allow virus to reemerge. Indeed, reduction of the acute inflammatory response may be essential, as products of activated CTLs and macrophages present within the lung may be sufficient to hinder variant replication. Experiments are underway to address these issues. Also of critical importance is the issue regarding CD₈⁺ T cell effector mechanisms in the selection of CTL escape variants. Studies on influenza pneumonia show that CD₈⁺ T cells can function in vivo via perforin/granzyme- or Fas/FasL-mediated cytolysis (53). Thus, it is of key interest to define the contribution of these CTL effector mechanisms in selection of CTL escape variants.

Sequence analysis of the CTL escape variants obtained in this study reveals a remarkable diversity in mutations within the NP gene of influenza A virus encoding the major H-2Dᵦ-restricted CTL epitope. The mutations are nonsynonymous and result in changes in the amino acid sequence. In this context, it is striking that pressure exerted by a monoclonal CTL population drives selection of variants that not only impair T cell recognition but also binding of peptide to the MHC molecule. The results in functional assays with variant peptides reveal that substitutions in the anchor residue at position 5 or 9 of NP366–374 peptide (374M→T, 370N→S, 370N→D, or 370N→I) gradually reduce the affinity of the peptide for H-2Dᵦ molecules. As expected, the peptide that contains alterations in TCR-exposed residues at position 6 (371M→I) binds efficiently to H-2Dᵦ without recognition by transgenic CTLs, whereas recognition by polyclonal CTLs is retained. Likewise, the peptide affinity of the variants containing the alteration 371M→V, 371M→I, or 372D→E was also tested and found to bind as well as wild-type peptide (data not shown).

The above observations are consistent with the three-dimensional structure of H-2Dᵦ-NP peptide complex (54), in which the main chain of the NP366–374 peptide at positions 5 and 9 is buried in the MHC groove, and residues 4, 6, and 7 are fully accessible to solvent. It is therefore likely that the unique in vitro pattern recognition by CTLs of target cells loaded with variant peptide (370N→S, 370N→D, or 370N→T) is a result of H-2Dᵦ-peptide ligand density rather than TCR ligand structure. That an excessive concentration of these variant peptides can induce F5 T cell proliferation and maturation into CTLs suggests that the signal emanating from TCR triggering on activated transgenic cells after engagement with H-2Dᵦ-variant peptide complexes is sufficient to initiate subsequent signal transduction events, resulting in cytotoxicity. In striking contrast, cells infected with the variant A/Mem/NP370S, A/Mem/NP370D, A/Mem/NP370T, or A/Mem/NP374T viruses do not achieve the MHC-peptide ligand density necessary for triggering T cell activation and effector function. The data described above can be interpreted within a model of T cell recognition based on both the total avidity contributed by TCR affinity for MHC and peptide, and the number of MHC-peptide complexes present at the cell surface. Thus, under the physiological conditions of infection with the variant A/Mem/NP370S, A/Mem/NP370D, A/Mem/NP370T, or A/Mem/NP374T viruses, the number of MHC and NP366–374 peptide complexes generated on the surface of APCs is not sufficient to trigger CTL activation. The virus with alteration 374M→T is particularly interesting, as this variant peptide possesses relatively high affinity to H-2Dᵦ compared with wild-type peptide, but no recognition of the virus occurs. One plausible explanation for this could be that the presence of threonine at the COOH terminus of the epitope affects peptide processing and presentation via the endogenous proteasome/transporter associated with antigen processing (TAP) transport pathway (55). Mutations in the sequence flanking the NP366–374 epitope that have been found to affect peptide transport or processing (56, 57) were not observed with the variants in this study, so this possibility can be formally excluded. Biochemical analyses are currently underway to test this hypothesis and define the specific factors for this blockage.

The significance of these epitope mutations was demonstrated in cytotoxicity and proliferation assays and by studying the polyclonal CTL response elicited in immunocompetent mice challenged with the variants. As approximately half of the bulk virus isolates with mutations in the anchor residue at position 5 or 9 could not be recognized by NP366–374 polyclonal CTLs, and no CTLs specific to NP were detectable in infected mice, the results suggest that these variants were biologically significant. The rest of the virus isolates contains changes in residues (e.g., position 6 and 7) not important for TCR binding. Therefore, it was not anticipated that changes in these residues would have a major effect on CTL recognition in immunocompetent animals, as a polyclonal CD₈⁺ CTL response should include TCRs with a wide spectrum of fine specificity for the NP366–374 epitope. However, epitope variants with mutations at TCR contact residues can function as agonists or antagonists of antigen-specific T cells both in vitro and in vivo (58–60). In addition, it has been reported for HIV, hepatitis B virus, and lymphocytic choriomeningitis virus that the order in which a host is exposed to virus variants can have significant effects on the outcome of infection. Thus, after initial exposure to a particular virus strain, reinfection with a variant bearing mutated TCR contact sites can boost the CTL response specific for the epitope peptide of the earlier infection (“original sin” for CTLs), or can an-
host antigen the pool of memory CTLs formed during the primary infection. The TCR contact site change in residue 7 of the NP366-374 epitope of 372D→E variant has been studied in detail in our previous report (61). Infection of F5 or F5-RAG-1 transgenic mice with X31 (a reassortant virus bearing an alteration [372D→E] within the NP366-374 epitope compared with A/M emphis/102/72) did not show transgenic CTL activation, and no specific cells were detected in the inflamed lungs. However, as the variant NP372D peptide has been described to antagonize F5 TCR activation (62, 63), we cannot exclude the possibility that this variant would be anergized in the draining LNs, preventing their recruitment to the lung. Similarly, in the case of A/M emphis/ NP371D, variant peptide displayed on the surface of APCs might antagonize a substantial part of NP366-374 epitope-specific CTLs in infected C57BL/6 mice or prevent CTL activation in F5 transgenic mice. However, it is unclear whether such variants could also be recognized efficiently by a memory T cell population generated after infection with the parental virus strain. Thus, it remains a challenge to test in a direct manner (by virus infection) how a polyclonal T cell repertoire would react during an encounter with different antigenic variants containing mutated TCR contact sites. It is tempting to speculate that exposure of a population to natural CTL escape variants, or selection of such variants during an influenza outbreak, could contribute to differences of severity, duration, or rarely, fatal spread of infection in infected individuals. Whether such variants could be perpetuated by viral transmission from host to host during an influenza outbreak is an open question.

Do CTL escape variants play a role in human influenza? Host-dependent selection pressure on influenza A virus surface antigens has been well defined, but the role of CTL responses in genetic drift at the epidemiological level is less clear. 2 of 11 known human influenza A virus CTL epitopes demonstrate a surprising degree of variation, which supports the view that even in acute infection, individuals may differ in their genetic susceptibility to viral escape from CTL selection pressure (64). Experimentally determined rates of virus gene/ protein mutation derived from field isolates probably are not representative of virus evolution within a single individual. Indeed, the example of persistent influenza A virus infection in immunodeficiency (65) demonstrates that antigenic changes can accumulate in the NP gene as rapidly as in the H A gene in vivo. Epidemiological studies stratified by MHC class I type or sequential isolates from acutely infected immunocompetent individuals of known class I type would be required to demonstrate naturally occurring CTL selection pressure. However, human CTL responses are often characterized by a limited number of dominant epitopes and restricted TCR usage, suggesting that at least some conditions favorable to CTL escape might occur in vivo. Thus, selection of CTL escape variants may play a part in normal individuals in a multistep process and/or under unusual conditions, such as nonspecific immunosuppression, either alone or in the context of T cell responses against one viral epitope or inefficient viral clearance by neutralizing antibodies. Whereas CTL escape variants may provide a survival benefit for the virus within an individual host, the effects of MHC polymorphism, polyclonality of the CTL response, and the existence of subdominant epitopes would have to be overcome for CTL escape variants to have a significant advantage within an outbred population. Further, early work showed that certain influenza NP-specific CTLs can recognize viral variants in vitro, and more recently it has been reported that TCRs cross-reactive between variants bearing mutations at residues involved in TCR binding can be induced infrequently in vivo (47, 48, 66). However, more work is required to explore whether such cross-reactive CTL responses can be induced in biologically significant levels upon exposure of a host to different variant viruses, and whether this would have consequences on the ability of the immune system to recognize and cope with the infection. Although studies in mice have demonstrated that influenza virus may still be cleared by antibody in the absence of a CTL response, studies to examine oscillation of CTL epitopes in the context of MHC type in sequential virus isolates from infected humans may prove worthwhile.

Persistence of influenza virus has been described in several immunocompromised children (65, 67). Although the duration varies (some individuals shed virus for months), prolonged infection characterized all the reported cases. However, viral persistence is not an absolute requirement for emergence and propagation of CTL escape variants. Our observations with transgenic mice reveal that substitutions within the NP366-374 epitope, which disrupts peptide binding to H-2Dβ and leads to loss of recognition by polyclonal CTLs, can be found frequently in the context of a dominant CTL response. The fact that the TCR repertoire in the transgenic mice is restricted to a particular effector cell type present at a high frequency does not necessarily diminish the relevance of these findings to a natural infection. Thus, it has been apparent, due to advances in methods for quantifying T cells, that influenza virus-specific CTLs can reach extremely high frequencies in normal mice and probably in humans (43). An accelerated virus-specific memory CTL response, for example, via immunization could force oscillations of CTL antigens, resulting in a prolonged course of infection. This would have significant impact on the clinical outcome of the disease.

In addition to the general interest in viral escape mechanisms, the realization that an important goal in vaccination may be the superior priming of T cells cross-reactive between different virus strains makes it necessary to understand CTL recognition in the context of antigenic variation of influenza virus. There are examples in the literature of both prophylactic and therapeutic strategies that have led to the selection of CTL escape variants (68, 69) mitigating the beneficial effects of such interventions. It is a key concern for future vaccine and/or therapy design to prevent a similar outcome.

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