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A broad cytotoxic T lymphocyte response to influenza type B virus presented by multiple HLA molecules

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Abstract

The HLA restriction and epitope specificity of cytotoxic T lymphocytes (CTL) involved in recovery from influenza type B infection have not been extensively characterized. Here lymphocytes obtained from a healthy individual contained virus-specific CTL restricted by class I HLA molecules, HLA-A1, A2, B7 and B8, and the class II HLA molecules, HLA-DR1 and DR3. Four conserved viral epitopes were predicted from allele-specific motifs for peptides interacting with HLA-B8 and HLA-DR1. Bulk CTL recognized three 9mer HLA-B8-restricted peptides from nucleoprotein, residues 30–38, 263–271 and 413–421, and a 13mer HLA-DR1-restricted peptide from hemagglutinin, residues 308–320. The epitopes presented by HLA-A1, HLA-B7 and HLA-DR3 remain undefined. Peptide-specific CTL lines recognized influenza type B virus-infected cells indicating the peptides are representative of naturally processed epitopes. A hemagglutinin peptide-specific CD4 CTL clone expressed ~200 molecules of perforin mRNA/cell, suggestive of a functional perforin pathway for target cell lysis. The results indicate a broad CTL response composed of both CD8 CTL and CD4 CTL recognizing viral epitopes presented by multiple HLA molecules.

Introduction

Each year the vaccine for influenza is updated to include the most current circulating strains of influenza type A and B viruses (1). Influenza type A is more heavily studied because it is the cause of pandemics. However, influenza type B frequently predominates in yearly influenza surveys. In the US, influenza type B epidemics occurred in approximately seven out of the last 15 years, including the winters of 1988–89, 1990–91 and 1992–93 (2–6). Complications arising from influenza type B infections include Reye's syndrome, respiratory failure, fatal rhabdomyolysis and encephalitis (7–10). While a major source of antigenic diversification in influenza type A is animal reservoirs, there are no animal reservoirs for influenza type B virus. Periodic epidemics of influenza type B virus appear to result from the accumulation of mutations selected by antibody-mediated immune pressure and co-circulation of distinct lineages of virus (6,11–19). Serological diversity due to changes in amino acid sequence is the major obstacle to effective vaccination against influenza B virus using the current killed vaccines that target B cell antibody responses. Identifying conserved epitopes in influenza B virus recognized by cytotoxic T lymphocytes (CTL) could contribute to the development of vaccines aimed at specifically generating CTL immunity.

CTL recognize viral peptides in the peptide-binding site of HLA molecules (20,21). Early CTL studies focusing on a single class I HLA molecule implied a CD8 CTL response directed against one dominant peptide. However, studies on the CTL response to HIV epitopes presented by HLA-B8 (22,23) suggest that multiple epitopes presented by a single HLA molecule compete for antigenic stimulation (24). One HLA-A2-restricted CD8 CTL epitope has been previously defined from influenza type B nucleoprotein (25,26). Here a broad CTL response composed of both CD8 CTL and CD4 CTL is demonstrated to influenza type B virus. Studies with whole virus indicated that most of the class I and class II HLA molecules expressed in the individual studied (HLA type: HLA-A1, A2, B7, B8, Cw7, DR1, DR3, DQw2, DQ5) presented viral CTL epitopes. Bulk CTL recognized four peptide epitopes predicted by known allele-specific motifs for peptides interacting with HLA-B8 and HLA-DR1. Peptides 30–38 and 263–

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Table 1. HLA type of CTL and target cells (HLA molecules expressed by the target cells shared with effector CTL are shown in bold)

CTL HLA type	**HLA-A1, A2, B7, B8, Cw7, HLA-DR1, DR3, DQw2, DQ5**
MANN	HLA-A29, B12, Cw4, Dw7, DR7
PR	HLA-**A1**, A24, B51, B62, Bw6, Bw4, DR11, DR13, DQ4
AT	HLA-**A2**, A11, B54, B60, Cw1, DR4, DR12, DQw3
PGF	HLA-A3, A3, **B7**, **B7**, DR2, DR2, DQ1, DP4
MGAR	HLA-A26, **B8**, Bw6, **Cw7**, DR15, DQ6, DP4
HOM-2	HLA-A3, A3, B27, B27, Bw4, C1 **DR1**, **DR1**, Dw4, **DQ5**
C3H L cell	HLA-**DR1**
MLB	HLA-A23, A30, B13, Bw50, Bw4, Bw6, **DR3**, DR7, Dw52, **DQw2**

271 from nucleoprotein presented by HLA-B8 and 308–321 from hemagglutinin presented by HLA-DR1 stimulated growth of CTL lines. Peptide 413–421 from nucleoprotein did not stimulate growth of a CTL line and was only consistently recognized by polyclonal CTL at the highest ratio. The amino acid sequences of all four peptides are generally conserved among viral isolates and thus can be classified as type B virus-specific CTL epitopes. The presence of both CD8 and CD4 CTL may have implications for vaccines aimed at boosting CTL responses.

Methods

Virus, viral peptides and viral nucleoprotein sequence

Influenza B/Hong Kong/1973 virus was grown in 10-day-old fertile chicken eggs incubated at 33°C for 2 days and harvested from allantoic fluid. Virus had a chicken red blood cell hemagglutinin titer of 1/128. Influenza B/Ann Arbor/1986 was a gift from William Biddison (NIH, Bethesda, MD). Vaccinia virus (ATCC, Rockville, MD) and recombinant vaccinia virus expressing influenza B nucleoprotein/Ann Arbor/1986 (25,27) were expanded in 143 B thymidine kinase minus human osteosarcoma cells (ATCC). The number of p.f.u./ml were determined on BSC-1 cells (ATCC). Viral peptides based on the sequences of nucleoprotein B/AA/86 (27), B/Lee/40 (28) and hemagglutinin B/HK/73 (13) were synthesized by the CBER Peptide Facility (Bethesda, MD), Tana Laboratories (Houston, TX) and Bio-Synthesis (Lewisville, TX). The molecular mass of peptides was checked by mass spectroscopy. Peptides were preincubated with target cells at 100 μg/ml. The nucleotide sequences of the nucleoprotein genes of the recent influenza B isolates, B/YM-88 and B/TX-88, were derived by direct sequencing of purified viral RNA as previously described (18). Sequencing primers were constructed based on the nucleoprotein sequence of B/AA/86 (27). The complete sequences of the nucleoprotein genes of B/TX and B/YM have been deposited at the Genome Sequence Data Base under accession nos L49384 and L49385 respectively.

CTL, target cells and CTL assay

The HLA type of the CTL and target cells used in these experiments are shown in Table 1. For generation of CTL and CTL lines, blood was collected from a healthy volunteer with a prior history of influenza infection. Ficoll-Hypaque separated white blood cells (1. 2×10^7) were infected with 40 μl of the influenza B virus/HK/73 stock in 8 ml RPMI for 1 h at 37°C in the absence of fetal bovine serum. After addition of fetal bovine serum (1 ml), cells were incubated for 5–7 days at 37°C in 5% CO_2 prior to testing for polyclonal CTL activity. CTL lines were generated from polyclonal CTL by repeated feeding with irradiated autologous B lymphoblasts pulsed with peptide (100 μg/ml). The CD4 CTL clone was obtained by dilution of the CTL line to 10 cells/well, with each well containing 5×10^4 irradiated allogenic peripheral blood lymphocytes, 5×10^4 irradiated autologous B lymphoblasts pulsed with 100 μg/ml of peptide, 1 μg/ml phytohemagglutinin (Wellcome, Research Triangle Park, NC) and 10 units/ml recombinant IL-2 (Cellular Products, Buffalo, NY). Target cells were Epstein–Barr virus-transformed B lymphoblasts or mouse L cells transfected with HLA-DR*B 0101 (Robert Ullrich, Research Institute of Infectious Diseases, USAM-RIID, Frederick, MD). Vaccinia virus infection of target cells was overnight at 37°C using 2 p.f.u./cell. The vaccinia virus used to make the recombinant, previously described (25), is the New York Board of Health strain. Influenza type B virus infection of target cells was 4 h prior to the assay using 100 μl of virus (HA titer 1/128). A 5 h CTL assay was used (25). Percent specific lysis was calculated from the equation $100\times$(experimental ^{51}Cr released – control ^{51}Cr release)/(maximum ^{51}Cr released – control ^{51}CR released). Experimental refers to effector cells with CTL activity together with non-lytic ^{51}Cr-labeled target cells, control refers to non-lytic ^{51}Cr-labeled target cells alone and maximum refers to ^{51}Cr-labeled non-lytic target cells in 5% Triton.

Quantitative-competitive PCR mRNA analysis

Total RNA was isolated from samples of $2–3\times10^6$ cells in 1 ml of TRIzol reagent (Gibco/BRL, Gaithersburg, MD) according to the manufacturer's instructions. SuperScript II reverse transcriptase (Gibco/BRL) was used with random hexamer priming, according to the manufacturer's instructions to prepare cDNA from total cellular RNA (1 μg per 20 μl reaction). DNA was isolated from Ficoll-Hypaque isolated PBMC by a standard SDS–proteinase K digestion technique. A 641 bp PCR product was generated using oligonucleotide primers from the 5′ region (GGA TTC CAG CTC CAT GGC AG) and from the complement of the 3′ region (GCT GGG TGG AGG CGT TGA AG) spanning two introns in the coding sequence of the human perforin gene (29). A 536 bp DNA competitive reference standard for this amplicon was prepared by the deletion method of Riedy *et al.* (30) using a 3′ region deletion primer (GGC GGG CTG GGT GGA GGC GTT GAA CCT GGT CCT GGT GGG TCT TCT). A 35 cycle, hot start PCR was performed on samples containing a fixed amount of cDNA and a series of 2-fold dilutions of reference standard, as per instructions in the GeneAmp kit (Perkin-Elmer Cetus, Branchburg, NJ) as modified by the manufacturer's instructions accompanying the AmpliWax beads (Perkin-Elmer Cetus). Optimum amplification of these amplicons was obtained with a reaction mix containing 0.75 mM $MgCl_2$ and PCR cycles consisting of a 1 min denaturation step (94°C) and a 1 min annealing step at 63°C followed by a 2.5 min

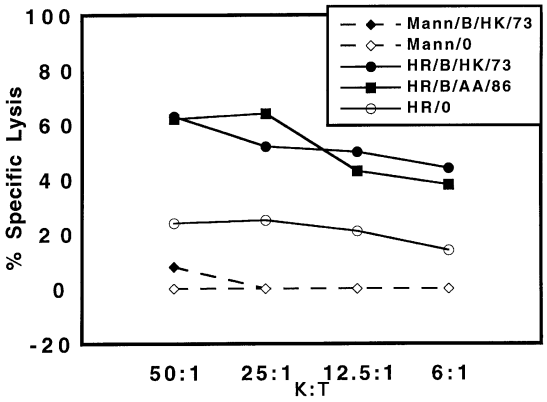


Fig. 1. Influenza type B-specific polyclonal CTL. Lysis of influenza B/Hong Kong/1973-infected HR autologous B lymphoblasts, influenza B/Ann Arbor/1986-infected HR autologous B lymphoblasts, uninfected HR autologous B lymphoblasts, influenza B/Hong Kong/1973-infected HLA-mismatched MANN B lymphoblasts and uninfected HLA-mismatched MANN B lymphoblasts.

extension step (72°C). A 597 bp PCR product was generated using oligonucleotide primers from the 5′ region (TTC CCT GTC CAA CCT CTG TG) and from the complement of the 3′ region (TCT TCC CCT CCA TCA TCA CC) spanning three introns in the coding sequence of the human Fas ligand gene (31). Optimum amplification of this amplicon was obtained in a 40 cycle PCR, annealing at 60°C with a reaction mix containing 0.75 mM $MgCl_2$. Amplified products were subjected to electrophoresis in a 2% (w/v) agarose gel containing 0.5 mg/ml ethidium bromide. After electrophoresis, video-images of PCR products were obtained with the UVP 5100 Gel Documentation System (UVP, San Gabriel, CA) and densitometric analysis of the quantity of PCR products was performed with NIH Image 1.55 software. The molar concentration of perforin mRNA in the samples was calculated by plotting the concentration of added reference standard versus the ratio of the band densities and determining the point of equivalence (30).

Results

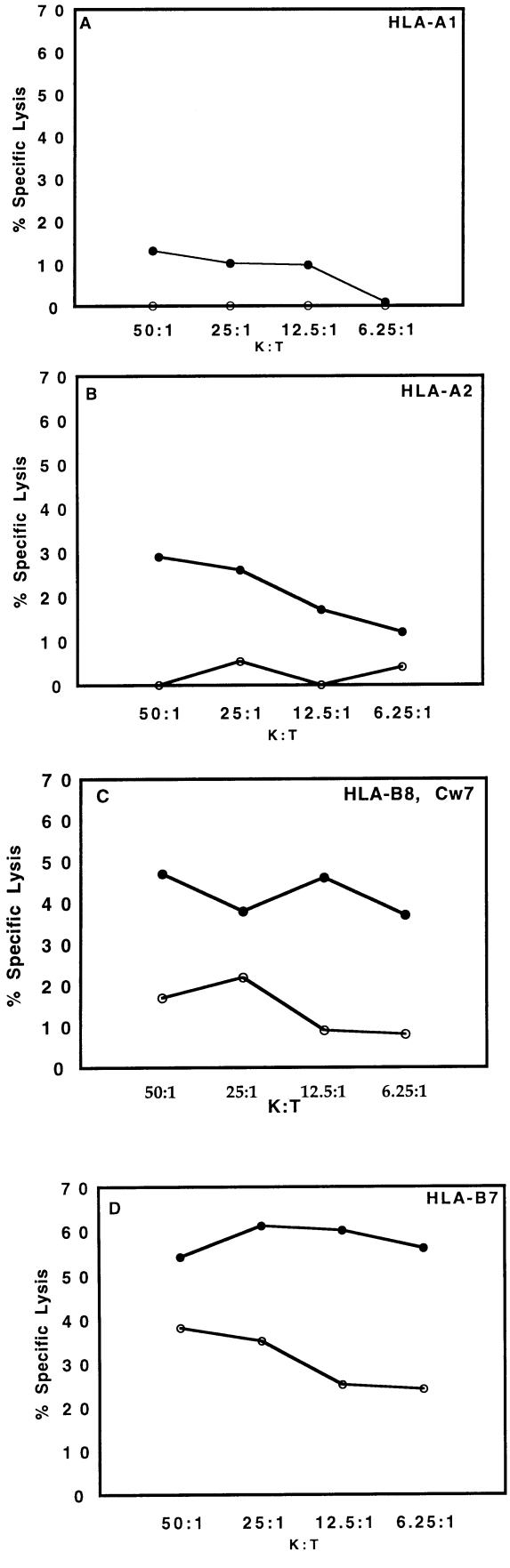
To determine whether the donor had memory CTL to influenza type B virus, peripheral blood mononuclear cells were stimulated with influenza B/HK/73 or influenza B/AA/1986 and tested 5 days later for detection of polyclonal CTL activity. CTL lysed autologous B lymphoblasts infected with either virus above that of uninfected cells, and did not lyse HLA-mismatched cells, MANN, either infected with B/HK/73 or uninfected (Fig. 1).

The class I HLA restriction of the response was determined using target cells sharing one or more class I HLA molecules with effector CTL (see Table 1). Polyclonal CTL lysed B/HK/73-infected PR B lymphoblasts sharing HLA-A1 (Fig. 2A) and AT B lymphoblasts B/HK/73-infected sharing HLA-A2 (Fig. 2B) better than uninfected cells. Polyclonal CTL also lysed B/HK/73-infected MGAR (Fig. 2C) and B/HK/73-infected PGF B lymphoblasts (Fig. 2D) better than uninfected cells. PGF shares HLA-B7 with the CTL, while MGAR shares HLA-B8 and Cw7. Thus, at minimum four class I HLA molecules, HLA-A1, HLA-A2, HLA-B7 and HLA-B8 and/or Cw7 presented peptide epitopes from influenza type B virus.

To determine if there was a class II-restricted CTL response to the virus, target cells sharing one or more class II HLA molecules with the donor were used (Table 1). Polyclonal CTL recognized HOM-2 cells infected with virus above that of cells uninfected (Fig. 3A). HOM-2 cells share HLA-DR1 and HLA-DQ5 with the effector CTL. To determine whether HLA-DR1 could present virus, polyclonal CTL were assayed for lysis of mouse C3H L cells transfected with HLA-DR1 infected and uninfected with B/HK/73 virus. CTL lysed L cells infected with virus, but not uninfected L cells (Fig. 3B). CTL also killed MLB cells infected with virus above that of uninfected MLB cells (Fig. 3C). MLB cells share HLA-DR3 and HLA-DQw2 with the CTL. Therefore, in addition to the presentation of virus by class I molecules, viral epitopes were presented by the class II molecules HLA-DR1 and HLA-DR3, and possibly HLA-DQ5 and DQw2.

Influenza virus expresses 10 proteins of which nucleoprotein, an abundant protein made during virus infection, is known to be a common target antigen for class I-restricted CTL (32). To determine whether viral nucleoprotein peptides were presented by HLA-B molecules, polyclonal CTL were tested for recognition of target cells infected with wild-type vaccinia virus and recombinant vaccinia virus expressing influenza B/AA/86 nucleoprotein. Polyclonal CTL recognized MGAR cells expressing type B nucleoprotein above MGAR cells infected with wild-type vaccinia virus (Fig. 4A), but had no recognition of nucleoprotein presented by PGF cells (Fig. 4A). Thus, HLA-B8 and/or HLA-Cw1 presented a peptide or peptides derived from nucleoprotein, while the peptides presented by HLA-B7 must derive from other viral proteins. Recognition of HLA-B8 could not be separated from HLA-Cw7 because the B8 haplotype in almost all instances is fixed in the HLA-B to DQ region.

The allele-specific motif for peptides interacting with HLA-B8 is known. These peptides often have an arginine (R) or lysine (K) in positions 3 and 5 and a leucine or isoleucine in position 9 (33,34). Defined CTL epitopes also reveal that isoleucine (I), leucine (L) or glutamine (Q) can be present at residues 3 or 5 (22,35,36). Examination of the amino acid sequence of influenza B/AA/86 nucleoprotein identified 28 possible peptides with residue R, K, L, I or Q at P3 and P5. Six peptides were chosen for synthesis based on their C-terminal residue. Five of the 28 sequences had a leucine or isoleucine at P8 or P9, and one had valine at P9. Two 9mer peptides 30–38 (RPIIRPATL) and 263–271 (ADRGLLRDI) were recognized by polyclonal CTL above background (Fig. 4B), while two 9mer peptides, 536–544 (SDKNKTNPI) and 481–489 (VRRMLSMNI), and one 8mer peptide, 377–384 (DAKDKSQL), were not recognized by polyclonal CTL above background (Fig. 4B and data not shown). The high non-antigen specific lysis could be due to NK or Epstein–Barr virus reactivity. The 9mer peptide, 413–422 (ALKCKGFHV), was recognized at a K:T ratio of 50:1 (Fig. 4B) and sporadically recognized at lower K:T ratios (data not shown), but repeated attempts to consistently stimulate growth of a CTL line using this peptide failed. CTL lines established on peptides 30–38 (Fig. 4C) and 263–271 (Fig. 4D) recognized both influenza type B virus-



infected and peptide-pulsed cells. The CTL line established with peptide 30–38 also recognized peptide 30–38(34K), the nucleoprotein amino acid sequence of the B/Lee/40 virus that has lysine instead of arginine at residue 34 (Fig. 4E).

In general, many of the peptides presented by class II HLA molecules derive from glycoproteins (37). Influenza B virus makes two glycosylated proteins, neuraminidase and hemagglutinin. In influenza type A virus, class II HLA molecules present peptides from these proteins (38–40). For peptides bound to HLA-DR1, tyrosines at position 1 (P1) of a core 9mer peptide and the third position from the N-terminus provide the primary anchor residues (41,42). Important secondary interactions occur at amino acid residues P4, P6 and P9 from the P1 tyrosine (42). Influenza B/HK/73 hemagglutinin had 12 possible peptide epitopes with tyrosine in the correct position. Of these possible epitopes, a 13mer peptide, residues 308–320, PYYTGEHAKAIGN, had the most compatible amino acid residues for the best secondary interactions with an alanine at P6 and an isoleucine at P9 (42). Polyclonal CTL lysed autologous (Fig. 5A) and HOM-2 B lymphoblasts (data not shown) pulsed with peptide 308–320 above that of target cells without peptide. A CTL line grown from these polyclonal CTL stimulated with peptide 308–320 recognized peptide 308–320 and influenza B virus presented on HOM-2 B lymphoblasts (Fig. 5B) and presented on autologous B lymphoblasts (data not shown). FACS analysis indicated this line was 85% $CD4^+$ (data not shown). An anti-HLA-DR mAb (L234), but not anti-HLA class I mAb (clone W6/32), inhibited recognition of the HA peptide 308–320 by this CTL line (Fig. 5C). The CTL line used in experiment Fig. 5(C) done at a later date after more re-stimulation with peptide contained 100% $CD4^+$ cells by FACS analysis (data not shown). A clone was isolated by limiting dilution from the hemagglutinin peptide-specific CTL line. FACS analysis indicated that this clone expressed CD4, not CD8 (data not shown). The clone lysed autologous target cells incubated with peptide 308–320 (Fig. 5D).

The CD4 CTL clone expressed perforin mRNA and Fas ligand mRNA (Fig. 6A). Quantitative-competitive PCR was performed to determine the level of perforin mRNA expressed in the CTL clone. From a plot of the data shown in Fig. 6(B) the point of equivalence is at 0.5 am for 2×10^{-3} μg total RNA; this calculates to be ~200 molecules of perforin mRNA/cell.

Discussion

The CTL response to influenza type B in one individual was examined in detail. A broad polyclonal response composed of both CD8 CTL and CD4 CTL to influenza type B was detectable after one *in vitro* stimulation and expansion of memory CTL by co-culture with virus. A minimum of five defined epitopes were recognized by bulk CTL; three peptide

Fig. 2. Class I HLA restriction of polyclonal influenza B CTL response. Lysis of influenza B/HK/73-infected (solid symbols) and uninfected (open symbols) PR B lymphoblasts sharing HLA-A1 (A), AT B lymphoblasts sharing HLA-A2 (B), MGAR B lymphoblasts sharing HLA-B8 and HLA-Cw7 (C), and PGF B lymphoblasts sharing HLA-B7 (D) with polyclonal CTL.

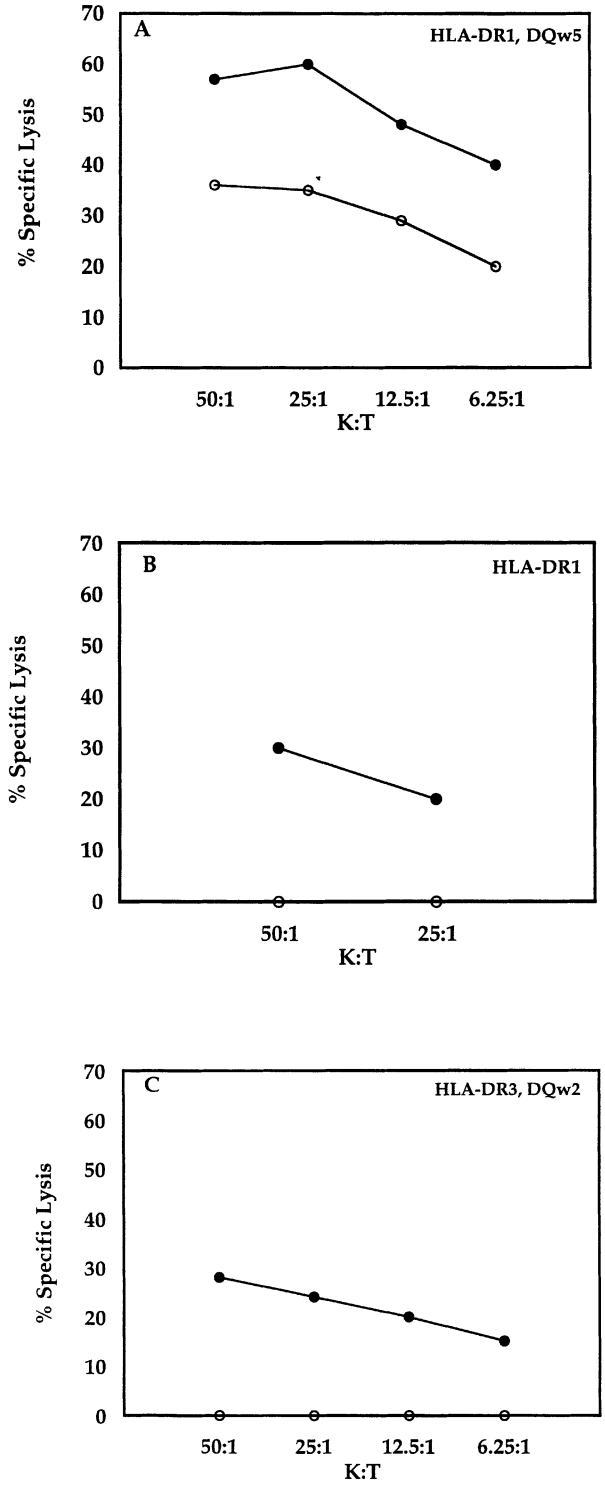


Fig. 3. Class II HLA restriction of the polyclonal influenza B CTL response. Lysis of influenza B/HK/73 (solid symbols) and uninfected (open symbols) HOM-2 B lymphoblasts sharing HLA-DR1 and HLA-DQ5 (A), C3H mouse L cells transfected with HLA-DR1 (B), and MLB B lymphoblasts sharing HLA-DR3 and HLA-DQw2 with the polyclonal CTL (C).

epitopes, 30–38, 262–271 and 413–421, from nucleoprotein presented by HLA-B8, one peptide epitope presented HLA-DR1 from hemagglutinin, 308–320, and an additional nucleoprotein epitope, 82–95, presented by HLA-A2 previously defined (25 and data not shown). Bulk CTL responses restricted by HLA-A1, B7 and DR3 indicate three additional epitopes bringing the minimum number of epitopes recognized by these polyclonal CTL to eight. Although the relative immunodominance among these epitopes was not determined, the CTL response appears directed against multiple epitopes and not a single epitope, since all of the epitopes indicated or defined were recognized by bulk CTL.

From the bulk CTL response it was apparent that among the epitopes presented by HLA-B8, CTL against nucleoprotein epitope 413–421 were less frequent than those against nucleoprotein epitopes 30–38 and 262–271. Moreover, of the six epitopes containing HLA-B8 binding motifs tested, only 30–38 and 262–271 were well recognized by bulk CTL. Peptide 413–421 was recognized only at the highest CTL concentration, while 377–389, 481–489 and 536–544 were not at all recognized, although all peptides had a HLA-B8 binding motif. The predicted 8mer peptide 377–389 might have been excluded from CTL recognition by its suboptimal length, which could favor the formation of unstable HLA molecules (26). Antigen-processing mechanisms, binding affinities or CTL repertorie must have favored CTL recognition of peptides 30–38 and 262–271 over peptides 413–421, 481–489 and 536–544.

Amino acid sequences of influenza type B nucleoprotein and hemagglutinin from different virus isolates were examined to determine whether the newly defined peptide epitopes were type, subtype or strain specific. The CTL epitope 263–271 from nucleoprotein presented by HLA-B8 was conserved among distinct influenza type B strains Lee/40, AA/86, SN/79, TX/88 and YM/88 differing in other parts of their nucleoprotein sequence (27,28,43). This epitope is conserved over time and conserved in viruses of distinct type B lineages, TX/88 and YM/88 (6,18,19). In the viral isolate B/Lee/40, the nucleoprotein residues 30–38 have a lysine at residue 34 (28), while SN/79, AA/86, TX/88 and YM/88 isolates have an arginine (27,28,43). However, either lysine or arginine serve as major anchor residues at P5 (which is residue 34 in the 30–38 peptide) for peptides binding to HLA-B8 (33,34) and consequently may not affect TCR recognition. Furthermore, the B/Lee/40 virus has not circulated since 1940 and was passaged extensively in mice before amino acid sequencing. Thus, although the epitope 30–38 differs in the B/Lee/40 isolate at P5, this substitution may not reflect the original isolate. However, to establish whether CTL specific for 30–38 (RPIIRPATL) recognized this substitution, peptide 30–38 (K34) representing the B/Lee/40 sequence was synthesized, tested and recognized by the 30–38(R34) CTL line. The sequence of nucleoprotein epitope 413–421 recognized sporadically and at high concentrations of bulk CTL is conserved in the viral isolates that differ in their nucleoprotein sequence (27,28,43). The HLA-DR1 hemagglutinin epitope (308–320) is conserved among distinct antigenic influenza type B isolates Lee/40, MD/59, HK/73, SN/79, SU/83, AA/86, VI/87, BJ/87, SH/87, YM/88, HK/88, SN/88, AI/88, TX/88 OH/88 and CD/88 (6,13,16,18) although hemagglutinin sequences from the 1988

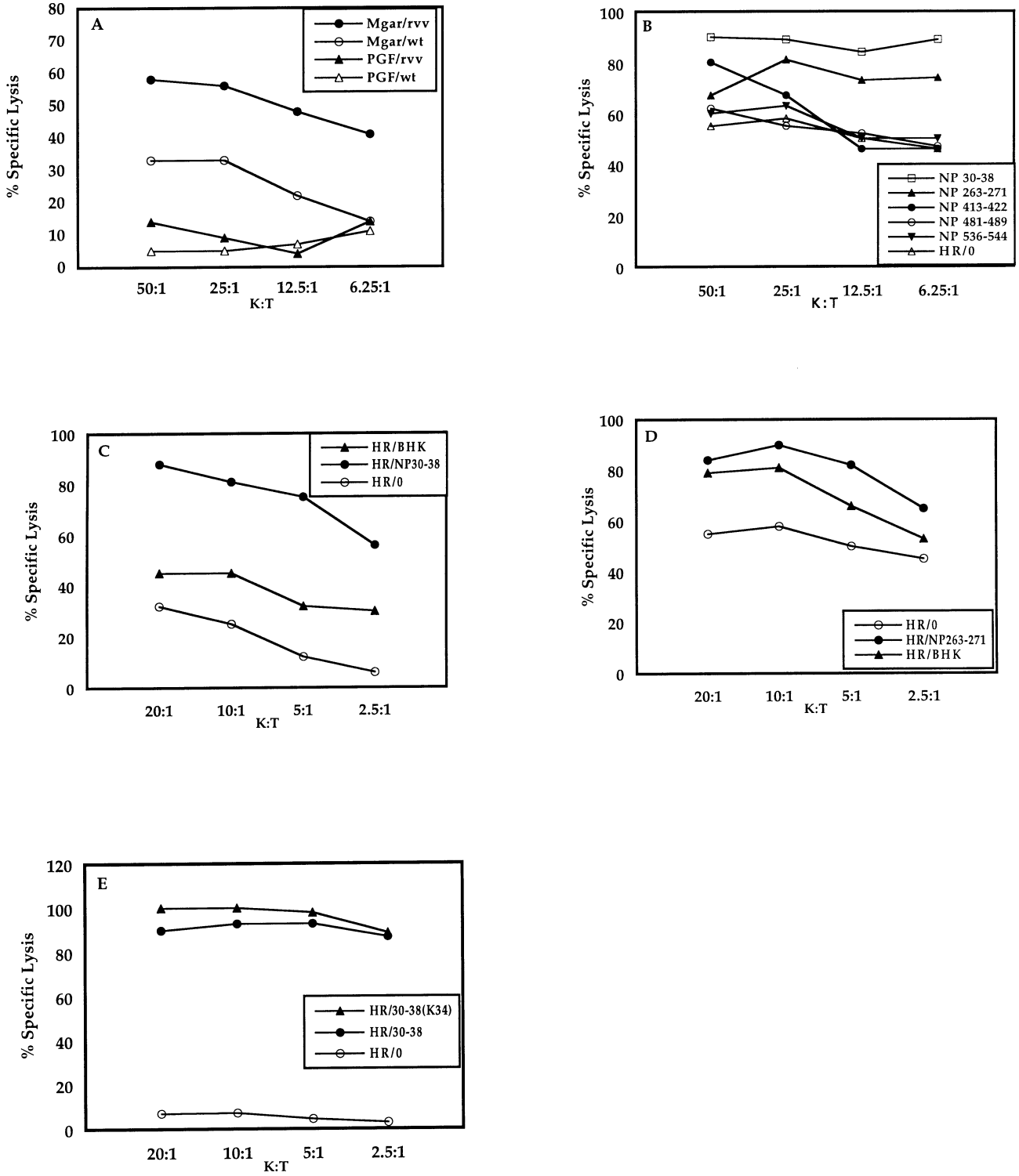
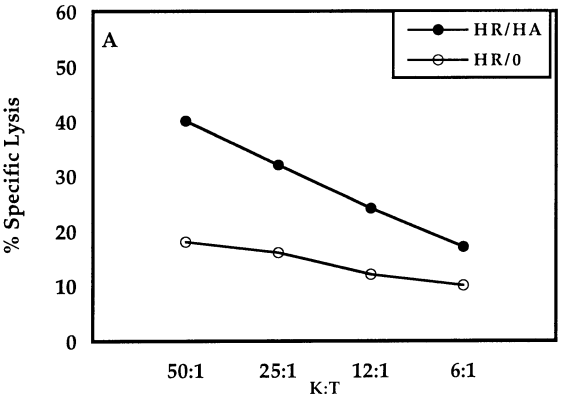
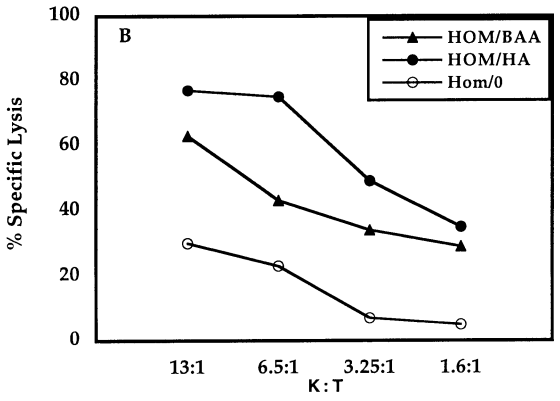
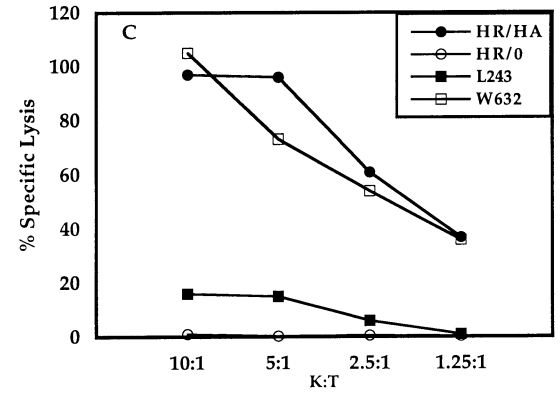


Fig. 4. CTL recognition of influenza type B nucleoprotein and nucleoprotein peptides. (A) Polyclonal CTL lysis of MGAR infected with either recombinant vaccinia virus expressing influenza B/Ann Arbor/1986 nucleoprotein (rvv) or wild-type vaccinia virus (wt) and of PGF infected with either recombinant vaccinia virus (rvv) or wild-type vaccinia virus (wt). (B) Polyclonal CTL recognition of peptides with HLA-B8 binding motifs from type B nucleoprotein presented by autologous B lymphoblasts. (C) CTL line recognition of autologous B lymphoblasts incubated with peptide 30–38, infected with influenza B/Hong Kong/1973 virus and uninfected. (D) CTL line recognition of autologous B lymphoblasts incubated with peptide 263–271, infected with influenza B /Hong Kong/1973 virus and uninfected. (E) CTL line recognition of peptide 30–38, peptide 30–38 (34K) and autologous B cells without peptide.







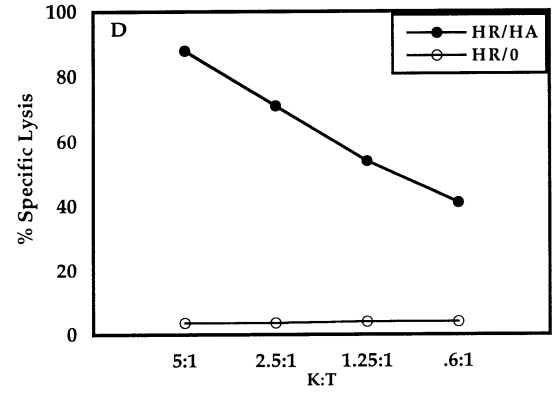


Fig. 5. CTL recognition of a 13mer peptide 308–320 from type B hemagglutinin. (A) Polyclonal CTL lysis of autologous B lymphoblasts incubated with 100 μg/ml peptide 308–320 or medium. (B) CTL line recognition of HOM-2 B lymphoblasts incubated with peptide 308–320, infected with influenza B/AA/86 and uninfected. (C) CTL line recognition of autologous B lymphoblasts incubated with HA peptide 308–320 in the presence of 1 mg/ml anti-HLA-DR mAb (clone L243), with HA peptide 308–320, with HLA peptide 308–320 in the presence of 1 mg/ml anti-class I HLA mAb (clone W6/32) and with medium. (D) CD4 CTL clone lysis of autologous B lymphoblasts incubated with peptide 308–320 or medium.

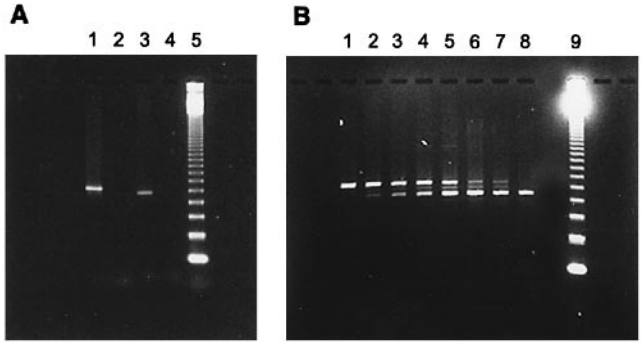


Fig. 6. PCR detection of perforin and Fas ligand sequences in mRNA. (A) Expression of perforin and fas in the CD4 CTL clone. Total RNA was isolated from the CTL clone, RNA was reverse transcribed with random hexamers and then cDNA was amplified with primers specific for human perforin (lane 1) and Fas ligand (lane 3) mRNA sequences. Normal human PBMC DNA was also amplified with primers specific for perforin (lane 2) and Fas ligand (lane 4). Lane 5 contains DNA mol. wt markers (123 bp DNA ladder; Sigma). (B) Agarose gel results of quantitative-competitive PCR for perforin mRNA expression in CD4 CTL clone. The amplifications shown in lanes 1–7 each contained cDNA derived from 1.5×10^3 proliferating cells. Amplifications also contained competitive reference standard DNA: 0.125 am (lane 2), 0.25 am (lane 3), 0.5 am (lane 4), 1.0 am (lane 5), 2.0 am (lane 6), 4.0 am (lanes 7 and 8). Lane 9 contains 123 bp ladder DNA mol. wt markers.

isolates differ by as much as 26% (6). Conservation of all of these peptide epitopes among sequenced isolates over time and over distinct viral lineages suggests that they are stable and therefore B type-specific CTL epitopes.

Are the conserved epitopes characterized intrinsically immunodominant or were they found because reactive CTL were disproportionately amplified by repeated viral exposure to different strains and *in vitro* stimulation? In dengue virus infections, cross-reactive CTL may be elicited during a secondary infection with a different serotype from the primary infection (44), but it is not known whether these CTL are protective. One of the first characteristics noticed about influenza type A-specific CTL was their cross-reactivity among different virus isolates (45,46). Subtype- and strain-specific CTL epitopes against influenza type A hemagglutinin have not been identified in polyclonal CTL responses, but only in cloned T cell populations (47). Subtype- and strain-specific CTL have not been identified against influenza type B proteins. Lower CTL frequencies for subtype- and strain-specific epitopes may make it difficult to detect such CTL. Whether CTL against conserved epitopes provide protection against

sequential infection or ameliorate the disease process with otherwise antigenically distinct viral isolates has not been tested.

Expression of the pore forming protein perforin, demonstrated in the CD4 CTL clone specific for a hemagglutinin peptide, indicates the similarity of CD4 CTL to CD8 CTL. Perforin is expressed in CD8 CTL and natural killer cells (48), but expression of perforin has not been found in all CD4 CTL (49–51). Fas ligand, a second method of target cell killing by cell-mediated cytotoxicity, is found in both CD8 and CD4 T cells (52,53). It has been suggested that CD4 CTL may rely more on the Fas ligand cytotoxic mechanism while CD8 cells, which are the more commonly studied cytotoxic T cells, use both perforin-mediated and Fas ligand mechanisms (53). The HA-specific CD4 CTL, described here, expressed both Fas ligand and perforin mRNA. Since perforin-mediated cytotoxicity by CD4 CTL was not expected, the amount of perforin mRNA per cell was determined to ascertain whether it was consistent with a functional level of perforin production. Using quantitative-competitive PCR analysis, we determined there to be ~200 molecules of perforin mRNA/cell in the HA-specific CD4 CTL clone; this represents moderate abundance for an mRNA and suggests significant perforin production, indicating a potential functional perforin-mediated cytotoxic pathway. Perforin pathways in both CD8 and CD4 T cells could indicate that either phenotype might function as CTL. CTL precursor frequency analysis using peripheral blood mononuclear cells from seropositive individuals indicate that CD8 and CD4 CTL exist in equal frequencies against chickenpox and parainfluenza viruses (54,55). *In vivo* evidence for CD4 CTL comes from β_2-microglobulin-deficient mice infected with lymphocytic choriomeningitis virus (56,57). However, the contribution of CD4 CTL against infectious organisms and intracellular antigen remains controversial, and only further experiments can help establish what the physiological importance of CD4 CTL are against intracellular pathogens.

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Abbreviation

CTL cytotoxic T lymphocyte

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