JOURNAL OF VIROLOGY, Nov. 1998, p. 8682–8689 Vol. 72, No. 11
0022-538X/98/$04.00+0

Human Cytotoxic T-Lymphocyte Repertoire to Influenza A Viruses

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Received 30 April 1998/Accepted 5 August 1998

The murine CD8$^+$ cytotoxic-T-lymphocyte (CTL) repertoire appears to be quite limited in response to influenza A viruses. The CTL responses to influenza A virus in humans were examined to determine if the CTL repertoire is also very limited. Bulk cultures revealed that a number of virus proteins were recognized in CTL assays. CTL lines were isolated from three donors for detailed study and found to be specific for epitopes on numerous influenza A viral proteins. Eight distinct CD8$^+$ CTL lines were isolated from donor 1. The proteins recognized by these cell lines included the nucleoprotein (NP), matrix protein (M1), nonstructural protein 1 (NS1), polymerases (PB1 and PB2), and hemagglutinin (HA). Two CD4$^+$ cell lines, one specific for neuraminidase (NA) and the other specific for M1, were also characterized. These CTL results were confirmed by precursor frequency analysis of peptide-specific gamma interferon-producing cells detected by ELISPOT. The epitopes recognized by 6 of these 10 cell lines have not been previously described; 8 of the 10 cell lines were cross-reactive to subtype H1N1, H2N2, and H3N2 viruses, 1 cell line was cross-reactive to subtypes H1N1 and H2N2, and 1 cell line was subtype H1N1 specific. A broad CTL repertoire was detected in the two other donors, and cell lines specific for the NP, NA, HA, M1, NS1, and M2 viral proteins were isolated. These findings indicate that the human memory CTL response to influenza A virus is broadly directed to epitopes on a wide variety of proteins, unlike the limited response observed following infection of mice.

Influenza A virus infections and complications are a major cause of human morbidity and mortality. Antibody responses to previous infection or vaccination are protective when the infecting strain is very similar to the vaccinating strain. However, the hemagglutinin (HA) and neuraminidase (NA) proteins undergo antigenic shift when these HA and/or NA genes reassort with a virus of a different subtype, thus evading antibodies (53). HA and NA can also undergo annual antigenic drift by accruing point mutations altering antibody binding sites (29, 37). It may be important for humans to have memory cytotoxic T lymphocytes (CTLs) in response to internal viral proteins, which are more conserved between viral subtypes, in view of the highly variable surface glycoproteins HA and NA on influenza A viruses, which can evade humoral responses. Virus-specific CTLs have been implicated in clearing influenza A virus infections in mice and humans (16, 24, 30–32, 34, 48, 54–56). CTLs specific to influenza A virus have been reported to be either subtype cross-reactive, recognizing targets infected with subtype H1N1, H2N2, and H3N2 influenza A viruses (24, 58, 59), or subtype specific, recognizing only the infecting subtype (6, 13). Bulk culture CTL responses specific to internal the influenza A virus nucleoprotein (NP), polymerases (PB1, PB2, and PA), and matrix protein (M1) have been reported in mice and humans (2, 3, 16, 42).

The repertoire of CTLs in response to influenza A viruses in inbred mice has been shown to be limited. It has been reported that there are low-responder or nonresponder class I alleles for influenza A virus CTL responses (3). Several murine class I alleles could not present epitopes to activate T cells on a number of viral proteins. The recognition of a viral protein by a major histocompatibility complex (MHC) allele was commonly found to be limited to immunodominant epitopes on one or two viral proteins (50, 52). In humans and mice, the CTL responses detected in bulk culture have been reported to be directed primarily at the NP (36, 49, 57). In a study of six human donors' peripheral blood mononuclear cells (PBMC) tested for cytotoxicity in bulk cultures stimulated by influenza A virus, all six recognized the NP, four recognized PB2, all six recognized M1, one recognized M2, and there was no recognition of the other viral proteins (16). It is important to define influenza viral protein and epitope recognition at the clonal level to determine if the human CTL response is restricted to a small number of proteins, as in the mouse system, or extends to a larger number of proteins and epitopes. Therefore, we analyzed in some detail the memory CTL repertoire in the PBMC of three human donors and found a very broad CTL response at the clonal level to epitopes on a wide variety of proteins.

MATERIALS AND METHODS

Viruses. Influenza A viruses A/Puerto Rico/8/34 (H1N1) and A/Japan/305/57 (H2N2) were kindly provided by the Division of Virology, Bureau of Biologics, Food and Drug Administration, Bethesda, Md. A/Johannesburg/94 (H3N2) was kindly provided by David Burt (Pasteur Merieux Connaught, Toronto, Ontario, Canada). Influenza A viruses were propagated in 10-day-old, embryonated chicken eggs. Infected allantoic fluids were harvested 2 days after infection, aliquoted, and stored at −80°C until use. Recombinant vaccinia viruses containing the genes coding for influenza A viral proteins HA, NA, M1, M2, PB1, PB2, PA, NS1, and NS2 and the nucleoprotein (NP) were kindly provided by B. Moss. They are all derived from the A/PR/8/34 influenza A virus strain, except for NS1, which is derived from A/Udorn/72. They were constructed and propagated as previously described (45). A recombinant vaccinia virus which expressed segmented portions of the NP was kindly provided by J. Bennink and L. Eisenlohr.

Human PBMC. PBMC specimens were obtained from normal, healthy donors. Most of the donors whose PBMC were tested had convincing evidence of influenza A virus-specific CTL activity in bulk culture. We concentrated our efforts on the PBMC of three donors from whom we were able to obtain repeat samples. PBMC were purified by Ficoll-Hypaque density gradient centrifugation (5). Cells were resuspended at 2×10^7/ml in RPMI 1640 with 20% fetal bovine serum (FBS) (Sigma) and 10% dimethyl sulfoxide and cryopreserved until use. The HLA alleles of donor 1 were A2.1, A11, B18, B27, Cw1, Cw7, DR1, DQw1,

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DQw3, DRw52, and DRw53; those of donor 2 were A2, A24, B7, B62, Cw3, DP2, DR1, DR2, DQw5, and DQw6; and those of donor 3 were A1, B8, B44, Cw5, DR2, DR3, DQw1, DQw2, and DRw52. HLA typing was performed in the HLA typing laboratory at the University of Massachusetts Medical Center.

Bulk cultures of PBMC. Responder PBMC were suspended at 10^6/ml in AIM-V medium (Gibco BRL, Grand Island, N.Y.) containing 10% human AB serum (NABI, Boca Raton, Fla.), penicillin-streptomycin, glutamine, and HEPES in a 70-ml flask (Falcon). Stimulators were infected with A/PR/8/34 at a multiplicity of infection (MOI) of 15 for 1.5 h at 37°C in 1 ml of phosphate-buffered saline containing 0.1% bovine serum albumin and then added to responders in a flask at a stimulator-responder ratio of 1:10. On day 7 of culture, cells were either cloned by limiting dilution as described below or restimulated with gamma-irradiated (3,000 rads) autologous PBMC infected with A/PR/8/34 at an MOI of 15 for 1.5 h in 1 ml of phosphate-buffered saline containing 0.1% bovine serum albumin, added at a stimulator-responder ratio of 1:10 in fresh medium containing 10% human AB serum and 20 U of interleukin-2 (IL-2) (Collaborative Biomedical Products, Bedford, Mass.). Restimulated cells were either cloned by limiting dilution or assayed for cytolytic activity 7 days later.

CTL clones. Influenza virus-specific CTL clones were established by using a limiting-dilution technique as previously described (22). PBMC which had been stimulated in bulk culture for 7 or 14 days were collected and plated at a concentration of 3, 10, or 30 cells per well in 96-well round-bottom microtiter plates in 100 μl of AIM-V medium containing 10% FBS, 20 U of IL-2, a 1:1,000 dilution of anti-CD3 monoclonal antibody 12F6 (kindly provided by Johnson Wong), and 10^5 gamma-irradiated allogeneic PBMC/well. On day 7, 50 μl of fresh medium with FBS (Sigma Immunochemicals, St. Louis, Mo.) and IL-2 were added, and on day 14, fresh medium with 10^5 gamma-irradiated allogeneic PBMC/well and a 1:1,000 dilution of the anti-CD3 monoclonal antibody were added. Growing cells were assayed for cytolytic activity on days 21 and 28. Cells from wells with influenza A virus-specific cytolytic activity were expanded to 48-well plates.

Preparation of target cells. Autologous lymphoblastoid cell lines (BLCL) were established by culturing with Epstein-Barr virus in 24-well plates as previously described (18). BLCL were infected with recombinant vaccinia viruses at an MOI of 20:1 for 1.5 h at 37°C. The cells were then diluted in 1 ml of medium and further incubated for 12 to 16 h. Other BLCL were infected with A/PR/8/34, A/Japan/305/57, or A/Johannesburg/94 in 1 ml of medium for 12 to 16 h. These infected target cells were labeled with 0.25 mCi of ^{51}Cr for 60 min at 37°C. After four washes, the target cells were counted and diluted to 2×10^4/ml for use in the cytotoxicity assay. The partially HLA-matched allogeneic target cells used in the assays were BLCL produced in our laboratory from the HLA-typed PBMC of unrelated donors or were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository or the American Society for Histocompatibility and Immunogenetics Cell Bank and Repository.

Cytotoxicity assays. Cytotoxicity assays were performed with 96-well round-bottom plates as previously reported (9). Briefly, effector cells in 100 μl of RPMI 1640 medium containing 10% FBS were added to 2×10^3 ^{51}Cr-labeled target cells in 100 μl at an effector-to-target (E-T) ratio of 10:1. In cytotoxicity assays using synthetic peptides, peptides were added to target cells at the indicated concentrations and incubated at 37°C for 30 min, after which the effector cells were added. Several of the M2, NS1, and NP peptides were kindly provided by Arthur Pedzcak and Pele Chong (Pasteur Merieux Connaught), and all other peptides were synthesized at the Core Protein Chemistry Facility directed by R. Carraway (University of Massachusetts Medical Center, Worcester). Plates were centrifuged at $200 \times g$ for 5 min and incubated for 4 to 5 h at 37°C. Supernatant fluids were harvested by using the supernatant collection system (Skatron Instruments, Sterling, Va.), and ^{51}Cr content was measured in a gamma counter. Percent specific ^{51}Cr release was calculated with the following formula: (cpm experimental release − cpm spontaneous release)/(cpm maximum release − cpm spontaneous release) × 100. All assays were performed in triplicate, and the results were calculated from the average of the triplicate wells.

Single-cell ELISPOT assay for IFN-γ-secreting cells. The ELISPOT assay was done as previously described (26). Briefly, 96-well filtration plates (Millipore, Bedford, Mass.) were coated with mouse anti-human gamma interferon (IFN-γ) antibody (clone NIB42; Pharmingen, San Diego, Calif.). Cryopreserved PBMC were thawed, washed, and added to the plates at 5×10^5 per well in RPMI 1640 medium supplemented with 10% FBS, penicillin-streptomycin, glutamine, and HEPES. Cells were incubated for up to 15 h with or without peptide stimulation (10 μg of peptide/ml). The plates were washed and then incubated with biotinylated mouse anti-human IFN-γ antibody (clone 4S.B3; Pharmingen). Spots were developed by using fresh substrate buffer (0.3-mg/ml 3-amino-9-ethylcarbazole and 0.015% H_2O_2 in 0.1 M sodium acetate [pH 5]). The precursor frequency of peptide-specific CTLs was calculated based on the number of spots counted out of the number of cells added to the wells.

RESULTS

Donor 1 PBMC in bulk culture exhibit influenza A virus subtype-cross-reactive lysis directed at multiple viral proteins. The PBMC from donor 1 were stimulated on days 0 and 7 with A/PR/8/34 (H1N1)-infected autologous stimulators and tested on day 14 against autologous BLCL infected with influenza A virus strains of each of the three subtypes (H1N1, H2N2, and H3N2). Uninfected BLCL were used as a negative control. The effector cells generated from the PBMC of donor 1 lysed targets infected with each of the influenza A virus strains to a higher degree than control uninfected targets (Fig. 1A).

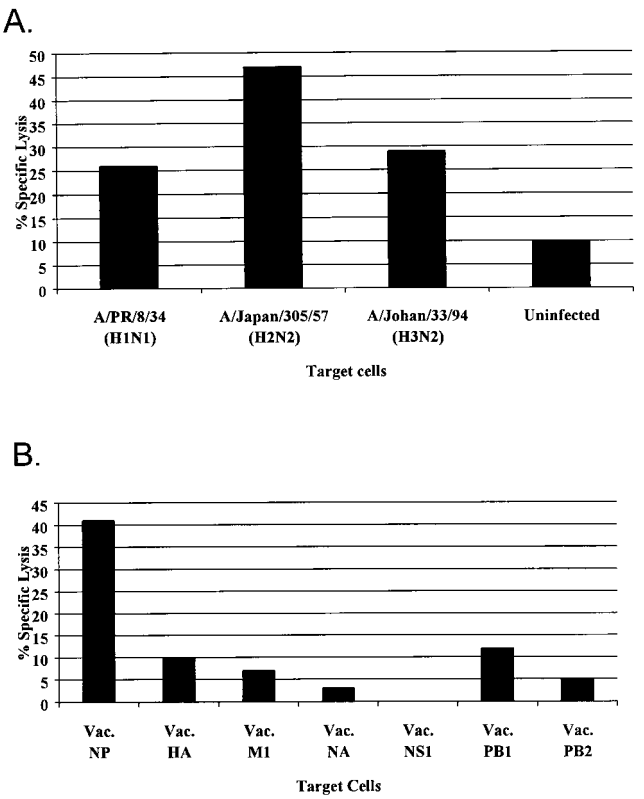

FIG. 1. (A) Influenza A virus subtype-cross-reactive lysis in bulk culture (donor 1). PBMC were stimulated with A/PR/8/34 virus in vitro on days 0 and 7 and tested on day 14 at an E-T ratio of 60:1. (B) Recognition of influenza A virus proteins in a bulk culture CTL assay (donor 1). The same bulk culture was tested for specific lysis of influenza viral proteins expressed in vaccinia virus (Vac.) constructs infected in BLCL at an E-T ratio of 60:1. Lysis of targets infected with wild-type vaccinia virus was subtracted from lysis of recombinant vaccinia virus-infected targets.

Recombinant vaccinia viruses were used to express various influenza A virus proteins in target cells tested with the same bulk culture. The highest level of influenza A virus-specific lysis was observed on NP-expressing target cells. Low levels of specific lysis were also seen on targets expressing PB1, HA, M1, and PB2 (Fig. 1B). In some experiments, low lysis of NA-expressing target cells was observed (data not shown).

Isolation of influenza A virus-specific CTL lines from donor 1. This bulk culture of influenza A virus-specific CTLs and subsequently two others from this donor were cloned by limiting dilution, and wells positive for growth were screened for lysis of influenza A virus-infected targets. All of the CTL lines were stained for CD4 or CD8 by fluorescent antibodies (data not shown). Ten CTL lines generated from the PBMC of donor 1 were tested for lysis of targets infected with the recombinant vaccinia viruses and influenza A virus (A/PR/8/34, H1N1). The results obtained with 6 of the 10 lines are presented in Table 1. These CTL lines have novel, previously unreported specificities. The other four cell lines characterized from this donor recognize epitopes that have been previously reported and are summarized below.

Each of the T-cell lines characterized was specific for one

TABLE 1. Identification of influenza virus proteins recognized by CTLs generated from PBMC of donor 1

Virus strain used to infect autologous BLCL	% Specific ^{51}Cr release[a]					
	CD8$^+$					CD4$^+$
	Expt 1, 10-1C4	Expt 2		Expt 3		Expt 4, 4-10D9-1
		10-1B7	1-2F8	4-30E11	10-1G5	
Influenza virus A/PR/8/34	42	46	29	12	50	86
Vaccinia virus HA				−1	70	
Vaccinia virus M1				26	−5	
Vaccinia virus M2				0	−1	
Vaccinia virus NS1				0	3	
Vaccinia virus NA		−1	6			37
Vaccinia virus NP	93					
Vaccinia virus PA		−1	2			
Vaccinia virus PB1		−1	68			
Vaccinia virus PB2		68	2			

[a] The E-T ratio was 10:1. Lysis of targets infected with wild-type vaccinia virus was subtracted from lysis of recombinant vaccinia virus. Lysis of the target cell by the CTL that demonstrates specific protein recognition is underlined.

influenza A virus protein (Table 1). These cell lines and those with previously reported specificities recognized the NP, PB2, M1, HA, PB1, NS1, and NA, making a total of seven different influenza A virus proteins recognized by the memory T lymphocytes of donor 1. These data indicate that the CTL responses of this donor to influenza virus are directed against a broad range of viral proteins and include both CD4$^+$ and CD8$^+$ components.

CTLs from donor 1 were HLA restricted. MHC restriction was assayed by using partially HLA-matched allogeneic BLCL as targets for CTL lysis. Targets were infected with the recombinant vaccinia virus that had resulted in significant target cell lysis or with wild-type vaccinia virus as a negative control. Alternatively, partially HLA-matched target cells were pulsed with a peptide that contained the CTL epitope. The results in Fig. 2A show that targets pulsed with NP peptide containing amino acids (aa) 173 to 193 are lysed by bulk culture effectors if they share HLA B27. Cell line 10-1C4 was subsequently found to lyse only target cells with B27 in common when pulsed with the same NP peptide, and it is therefore also B27 restricted (data not shown). Cell lines 10-1B7 and 1-2F8 are also able to lyse only targets which share B27 and are therefore B27 restricted (Fig. 2B and C). Cell line 4-30E11 is either A11, Cw7, or Cw1 restricted, because it does not lyse targets expressing A2, B27, and B18 (Fig. 2D). This cell line ceased growing and could not be tested further. HA-specific cell line 10-1G5 is B18 restricted because it lyses targets which have only B18 in common (Fig. 2E). We also tested CD4$^+$ T-cell line 4-10D9-1, which is DR1 restricted (data not shown) and another four T-cell lines which recognized previously reported epitopes and were found to be restricted by HLA A2.1, B27, and DR1 (data not shown) (17, 20, 33, 44). The results shown in Fig. 2 are a representation of the experiments done to confirm MHC restriction of the CTL lines. MHC restriction of each cell line was confirmed in multiple experiments using different allogeneic targets. A2.1-restricted lines were confirmed by lysis of infected Hmy C1R A2.1-transfected targets. These results indicate that at least four different class I HLA alleles (A2.1, B18, B27, and A11, Cw1, or Cw7) and one class II (DR1) HLA allele restrict the CTL lines isolated from donor 1, and there does not seem to be one influenza A virus-specific dominant HLA allele.

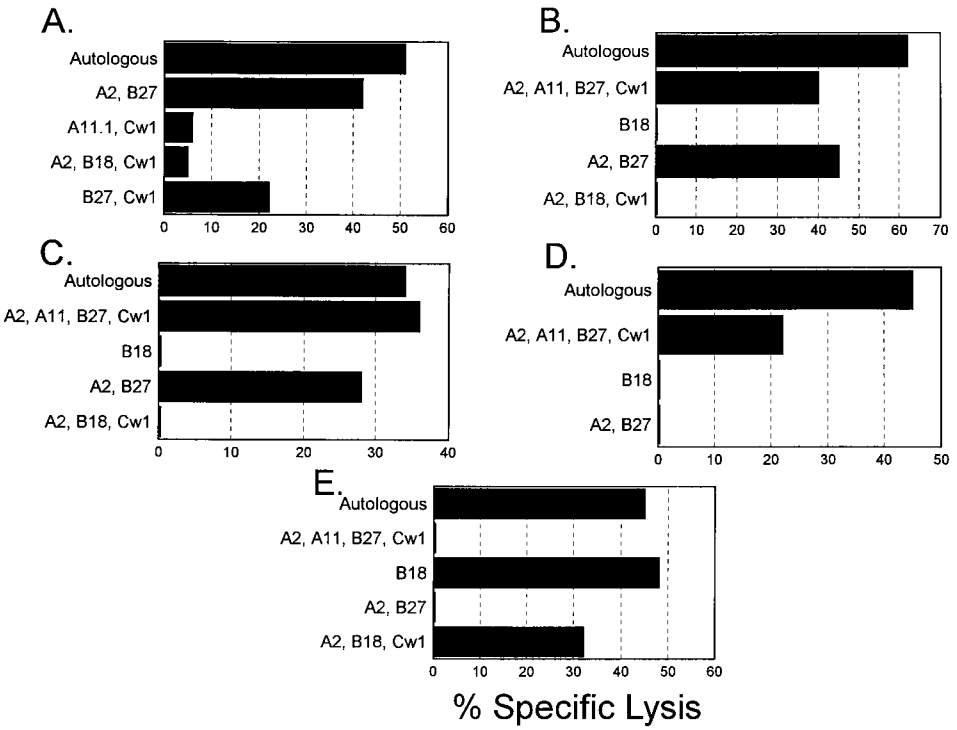

FIG. 2. MHC restriction of cell lines determined by using panels of partially HLA-matched allogeneic targets infected with vaccinia virus recombinants expressing either an influenza virus protein or a specific peptide. Lysis of targets infected with wild-type vaccinia virus was subtracted from lysis of recombinant vaccinia virus-infected targets. (A) Bulk culture cells tested against targets loaded with the NP aa 174 to 184 peptide. (B) Cell line 10-1B7 tested against vaccinia virus PB2-infected targets. (C) Cell line 1-2F8 tested against vaccinia virus PB1-infected targets. (D) Cell line 4-30E11 tested against vaccinia virus M1-infected targets. (E) Cell line 10-1G5 tested against vaccinia virus HA-infected targets. The E-T ratio was 10:1, except for panel A, for which the E-T ratio was 30:1.

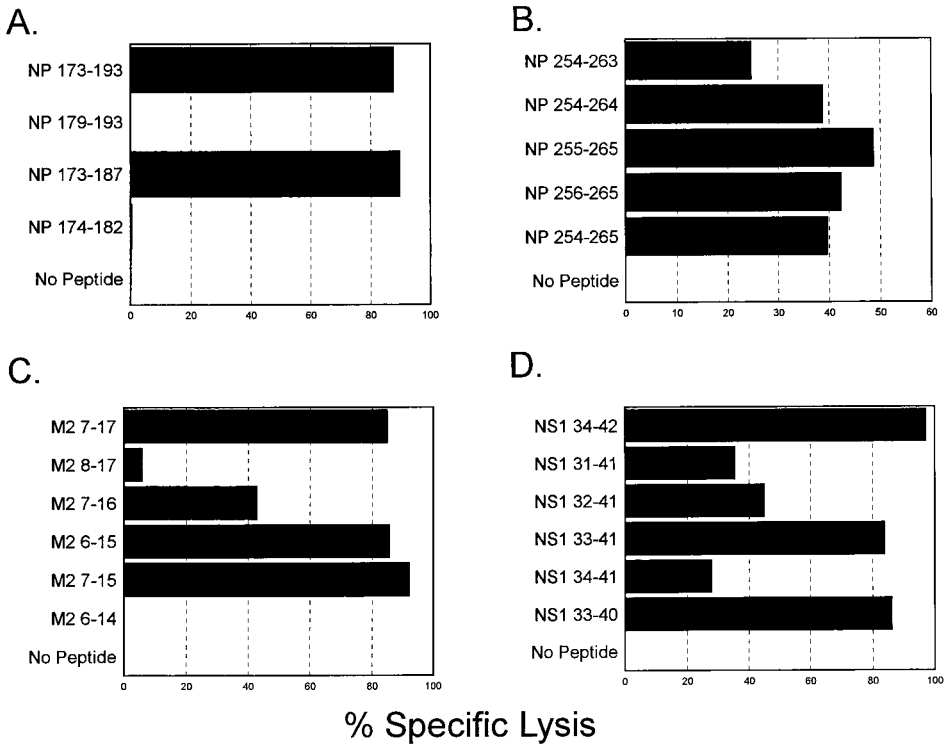

FIG. 3. Mapping of CTL epitopes by using peptides. The epitopes recognized by cell line 10-1C4 from donor 1 (A), 3E5 from donor 2 (B), and 124 (C) and 77 (D) from donor 3 were mapped by using peptide-pulsed BLCL targets at a concentration of 25 μg/ml. The E-T ratios were 10:1 (A and B), 7.5:1 (C), and 15:1 (D).

T-cell epitope mapping. Recombinant vaccinia viruses were used that contain overlapping amino acid regions of the NP gene (aa 1 to 168, 147 to 315, and 296 to 498) to localize the epitope recognized by NP-specific cell line 10-1C4. This cell line lysed targets infected with the construct expressing NP aa 147 to 315. We synthesized 20-mer peptides that spanned aa 147 to 315, and this cell line lysed targets pulsed with a peptide containing aa 173 to 193. Target cells pulsed with this peptide were also recognized by effector cells in a 7-day bulk culture from this donor (data not shown). Finer mapping with synthetic peptides indicated that the optimal epitope is contained within aa 174 to 184 (Fig. 3A and 4A). Precursor frequency analysis by ELISPOT single-cell IFN-γ secretion indicated that this CTL epitope is recognized by 1 in 4,156 donor 1 PBMC (Table 2).

Peptides containing epitopes that were previously reported were synthesized and tested for recognition by these CTL lines if they shared MHC restriction and viral protein specificities with the cell lines we isolated. Cell line 10-2C2 lysed targets pulsed with a peptide representing aa 122 to 130 of NS1, line 1-7-K lysed targets pulsed with peptide aa 58 to 66 of M1, line 1-3 lysed targets pulsed with peptide aa 17 to 31 of M1, and line 1-1 lysed targets pulsed with peptide aa 383 to 391 of NP. Precursor analysis of these epitopes in this donor's PBMC confirmed that these are not rare CTLs generated by the cloning process (Table 2).

Donors 2 and 3 also have broad CTL repertoires for influenza A viral proteins. After finding a broad repertoire of CTL responses to influenza A virus in donor 1, we wanted to analyze the PBMC of other donors in a limited way to determine if they also had broad CTL responses. Bulk culture CTL assay results obtained by using PBMC from donor 2 revealed a similar pattern of influenza A virus-specific lysis with higher lysis of the NP and a lower level of NS1-specific lysis (data not shown). A bulk culture was cloned to identify protein recognition at the clonal level. After one limiting dilution, five different cell lines were shown to have specific lytic activity against four different viral proteins (HA, NP, NA, and M1). Three $CD4^+$ lines and two $CD8^+$ lines were characterized (Table 3). There were two NP-specific cell lines, $CD8^+$ 3G11 and $CD4^+$ 3E5. By using recombinant vaccinia viruses expressing the segmented NP, 3G11 lysed targets infected with vaccinia virus NP aa 296 to 498, and 3E5 lysed targets infected with vaccinia virus NP aa 147 to 315. Fine epitope mapping using synthetic peptides demonstrated that cell line 3E5 recognizes NP aa 256 to 265 at 0.25 mg/ml (Fig. 3B and 4B). Both cell lines 3F4 and 3G11 were found to be B62 restricted (data not shown) by lysis of partially HLA-matched targets as described for donor 1. The restricting alleles of the three $CD4^+$ CTL lines were not determined because of failure to lyse available partially matched BLCL targets.

PBMC from donor 3 were also tested in a single bulk culture CTL assay, and specific NP, NS1, and M2 recognition was seen (data not shown). This bulk culture was cloned, and two influenza virus-specific cell lines were established. Cell line 124 is $CD8^+$ and recognizes M2, while cell line 77 is $CD4^+$ and recognizes NS1 (Table 3). Synthetic peptides were used to identify the epitopes. Cell line 124 recognized aa 7 to 15 of M2 (Fig. 3C and 4C), while cell line 77 recognized aa 34 to 42 (Fig. 3D and 4D). HLA restriction analysis was performed by using allogeneic partially HLA-matched cell lines as done with the two previous donors; CTL line 124 is restricted by B44, and CTL line 77 is restricted by DR3 (data not shown).

Subtype cross-reactivity of CTLs isolated from all donors. It has been previously shown that influenza virus-specific memory CTL in mice are either subtype specific or cross-reactive

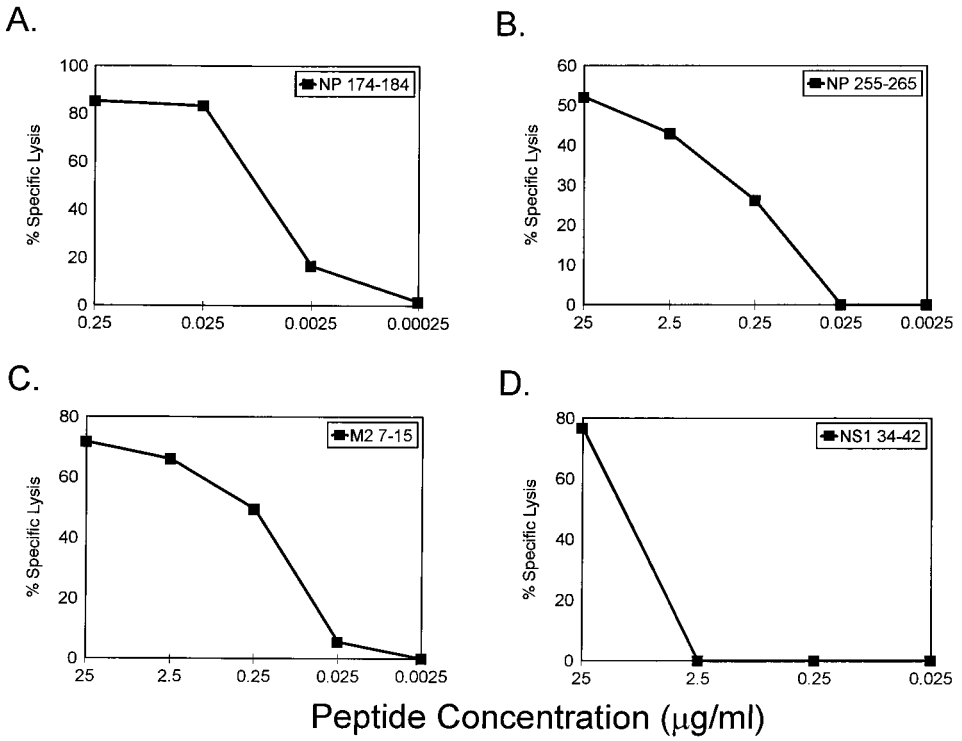

FIG. 4. Dose response of peptide-pulsed target cell lysis. Cell line 10-1C4 from donor 1 (A), 3E5 from donor 2 (B), and 124 (C) and 77 (D) from donor 3 recognized target cells pulsed with various peptide concentrations. The E-T ratios were 10:1 (A and B) and 15:1 (C and D).

(6, 13, 24, 58, 59). After stimulating PBMC with A/PR/8/34 (H1N1), we infected targets with viral strains of the three different subtypes (H1N1, H2N2, and H3N2) to analyze the subtype-cross-reactive nature of these T-cell lines. In all donors, most of the cell lines that had specificities to internal viral proteins (10-1C4, 10-1B7, 4-30E11, 1-2F8, 3G11, 10E7, 77, and 3E5) were H1N1, H2N2, and H3N2 subtype cross-reactive (Table 4). Cell lines with specificity to the external glycoproteins (10-1G5, 4-10D9-1, 3F4, and 3E9) were either H1N1 and H2N2 subtype cross-reactive or H1N1 subtype specific. In donor 1, HA-specific cell line 10-1G5 demonstrated H1N1- and H2N2-cross-reactive killing (Table 4). Cell line 124 is unique in that it is specific for conserved internal protein M2 and is H1N1 and H2N2 but not H3N2 cross-reactive. These results indicate that these donors have both subtype-specific and cross-reactive CTLs.

DISCUSSION

In this study, we analyzed the influenza virus-specific T-cell repertoire of three healthy adults. These individuals had bulk CTL responses to multiple influenza A virus proteins. Previous reports identified the NP, M1, and PB2 proteins as targets for human influenza A virus-specific CTLs in bulk culture (16). The NP was recognized by the T cells of all donors, but PB1- and HA-expressing targets were also lysed by the bulk culture T cells of donor 1, and NS1-expressing target cells were also lysed by donor 2 and 3 T cells in bulk culture. In view of the fact that influenza A virus-stimulated bulk cultures recognized multiple influenza A virus proteins, we decided to define CTL epitopes at the clonal level. Previously, a narrow CTL response to influenza A virus had been reported in mice (3, 41, 52). For example, targets infected with a vaccinia virus expressing the NP were lysed by bulk culture T cells from virus-immune mice with the K^k or K^d allele but not by the T cells of mice with the D^k, D^d, or L^d allele. Moreover, targets infected with a vaccinia virus expressing PB2 were lysed by T cells only from mice with the D^d allele (3). Some nonimmunodominant epitopes have been reported in the *H-2*^{b} mouse. They were derived by multiple peptide immunizations and were not detected in mice after infection with virus (39, 40).

Human CTLs that recognize influenza A virus-infected targets have been described, but most reports have focused on a single HLA allele, and T-cell lines were not usually isolated from the same donor (11, 12, 17, 20, 33, 35, 47). There has been a report of CTLs that recognize multiple NP epitopes on influenza B virus in one human (43), but limited information is available on influenza A virus-specific human T-cell epitopes. $CD8^+$ clones specific to the NP (11, 12, 20, 35, 47), M1 (17), PB1 (12), and NS1 (33) have been characterized, and $CD4^+$ clones specific to HA (8, 27), the NP (10), NA (46), and M1 (44) have been identified. Human CTL epitopes have also

TABLE 2. Peptide specific CTL frequencies in donor 1 PBMC as determined by ELISPOT[a]

Influenza virus protein	Amino acids	MHC class I restriction	Precursor frequency of peptide-specific IFN-γ spot-forming cells[b]
NP	174–184	B27	1/4,156
M1	58–66	A2.1	1/31,250
NP	383–391	B27	1/16,447
NS1	122–130	A2.1	<1/500,000
M1	17–31	DR1	1/26,316

[a] Controls included addition of no peptide or an irrelevant peptide.
[b] The results of this experiment are representative of those observed in three experiments using this donor's PBMC.

TABLE 3. Recognition of influenza virus proteins by CTL lines generated from the PBMC of two other donors

Virus strain used to infect autologous BLCL	% Specific ^{51}Cr release[a]						
	Expt 1, 3F4, CD8$^+$	Expt 2		Expt 3		Expt 4[b]	
		3G11, CD8$^+$	3E5, CD4$^+$	3E9, CD4$^+$	10E7, CD4$^+$	124, CD8$^+$	77, CD4$^+$
Influenza virus A/PR/8/34	36			75	56	27	72
Vaccinia virus HA	52[c]					−6	−4
Vaccinia virus M1	−2			0	66		
Vaccinia virus M2	−10					69	1
Vaccinia virus NA	0			59	2	0	2
Vaccinia virus NP		65	65			−2	0
Vaccinia virus NP aa 1–168		−2	−2				
Vaccinia virus NP aa 147–315		1	33				
Vaccinia virus NP aa 296–498		67	−6				
Vaccinia virus PA				0	0		
Vaccinia virus PB1				−1	−1		
Vaccinia virus PB2				−3	−1	1	−1
Vaccinia virus NS1						3	74

[a] The E-T ratios was 10:1. The lysis of targets infected with wild-type vaccinia virus was subtracted from the lysis of targets infected with recombinant vaccinia viruses.
[b] These T-cell lines were from donor 3; all others were from donor 2.
[c] Lysis of the target cell demonstrating specific protein recognition by the cell line is underlined.

been mapped on multiple proteins in human immunodeficiency virus type 1 infection. However, most of those CTL epitopes were also mapped in different individuals (21). Human immunodeficiency virus infection is persistent and may induce a different pattern of CTL responses than an acute self-limited infection with influenza A virus.

Many influenza A virus-specific T-cell lines were isolated from these three donors, and some were characterized in more detail. In donor 1, of the 10 lines characterized, seven different viral proteins were recognized: NP, NS1, HA, PB1, PB2, M1, and NA. These results reflect a broad pattern of T-cell recognition of epitopes on multiple influenza virus proteins. A larger number of cell lines isolated from this donor were not studied in detail but also reflected this very broad CD8/CD4 CTL recognition of multiple influenza A virus proteins (data not shown). To our knowledge, this is the first report of human CD8$^+$ CTL clones that recognize epitopes on the PB2 and HA proteins of influenza A virus. From donor 2, five cell lines were found to be specific for four different viral proteins: HA, the NP, NA, and M1 (Table 3). Two cell lines from the PBMC of donor 3 were specific for two different proteins, NS1 and M2 (Table 3). This is the first characterization of a human CD8$^+$

TABLE 4. Subtype-cross-reactive and -specific recognition by influenza virus-specific CTLs from donors 1, 2, and 3

Cell line[b]	Protein specificity	Epitope (amino acids)	CD4/CD8	% Specific ^{51}Cr release[a]		
				A/PR/8/34, H1N1	A/Jap/305/57, H2N2	A/Johan/33/94, H3N2
H1N1, H2N2, and H3N2 subtype-cross-reactive CTL lines						
10-1C4	NP	174–184	CD8	18	46	12
10-1C4	NP	174–184	CD8	22	ND[c]	17
10-1C4	NP	174–184	CD8	15	ND	15
10-1B7	PB2		CD8	25	41	30
10-1B7	PB2		CD8	46	60	ND
4-30E11	M1		CD8	25	53	ND
1-2F8	PB1		CD8	13	49	20
1-2F8	PB1		CD8	16	56	ND
3G11	NP	315–496	CD8	56	86	40
10E7	M1		CD4	53	78	65
3E5	NP	254–264	CD4	97	95	87
77	NS1	34–42	CD4	82	64	ND
77	NS1	34–42	CD4	ND	ND	76
H1 and H2 subtype-cross-reactive and H1 subtype-specific CTL lines						
10-1G5	HA		CD8	32	64	−7
10-1G5	HA		CD8	40	48	ND
4-10D9-1	NA		CD4	44	8	−1
3F4	HA		CD8	55	2	−3
3E9	NA		CD4	62	13	9
124	M2	7–15	CD8	69	55	5

[a] The E-T ratio was 10:1. Lysis of uninfected targets was subtracted from that of influenza virus-infected targets.
[b] Some cell lines were tested in multiple experiments. The same experiment may have included more than one cell line.
[c] ND, not tested in that experiment.

CTL line specific for an epitope on M2 and a $CD4^+$ CTL line specific for NS1. Both $CD8^+$ and $CD4^+$ CTL lines were isolated from each of the donors. Our results suggest that humans have memory CTLs that respond to multiple epitopes on several viral proteins and that there is no single immunodominant epitope, as reported in the murine system for influenza and other viral diseases (19, 51, 52). Due to the antigenic variation of influenza A virus strains, it may be beneficial for a human host to develop a polyclonal response to ensure the ability to clear influenza virus-infected cells despite mutations in the surface glycoproteins.

The cell lines from donor 1 were restricted by several different HLA alleles, including A2.1, B27, B18, and DR1 (Fig. 2). Donor 2 had cell lines restricted by B62 and a class II allele, while donor 3 had cell lines restricted by B44 and DR3. HLA alleles were found to present multiple epitopes on more than one viral protein, e.g., HLA B27-restricted epitopes on PB1, PB2, and the NP in donor 1. HLA A2.1 molecules presented peptides in both M1 and NS1. Similarly, in donor 2, B62 molecules presented epitopes on the NP and HA.

To verify that the cell lines we isolated were not rare, precursor frequency analysis was determined by using ELISPOT single-cell IFN-γ secretion to detect peptide-specific CTLs. Other groups have used this method to detect influenza virus-specific precursor CTLs in humans (26) and lymphocytic choriomeningitis virus (LCMV)-specific precursor CTLs in mice (38). In donor 1, the most precursor CTLs were directed at NP aa 174 to 184, with a frequency of 1 in 4,156. However, M1 aa 58 to 66, M1 aa 17 to 31, and NP aa 383 to 391 had precursor frequencies of 1 in 31,250, 1 in 26,316, and 1 in 16,447 respectively, indicating that they are also not rare CTLs (Table 2).

Subtype-cross-reactive and subtype-specific $CD8^+$ CTLs have been isolated from mice (4, 7, 25), and subtype-cross-reactive $CD8^+$ CTLs have been isolated from humans (35). In humans, subtype-cross-reactive and subtype-specific lymphocyte proliferation was previously reported (28). The CTL lines we characterized from our three donors were usually H1N1, H2N2, and H3N2 subtype cross-reactive if they recognized epitopes on relatively conserved internal viral proteins, such as the NP, NS1, PB1, PB2, and M1 (Table 4). These results are consistent with the fact that internal proteins are more conserved than HA and NA. Memory CTLs to internal conserved viral proteins may be protective when a different subtype of influenza A virus infects. Cell lines were also found that were either H1N1 and H2N2 subtype cross-reactive or H1N1 subtype specific if the CTL line recognized epitopes on the outer, more variable glycoproteins HA and NA (Table 4). Hemagglutinins H1 and H2 are much closer in homology than H1 and H3. An H1-H2-cross-reactive $CD8^+$ epitope on the HA transmembrane has been reported in *H-2*^{d} mice (24). A previous report identified human $CD4^+$ T-lymphocyte clones that proliferated in response to HA, NA, M1, and NP (28), and $CD4^+$ HA and NA subtype-specific CTLs have been identified (46), but to our knowledge, this is the first report of HA subtype-specific human $CD8^+$ CTLs.

We isolated and defined six $CD4^+$ CTL lines. The role of $CD4^+$ CTLs in virus infections, including influenza, is not as well defined as is the role of $CD8^+$ CTLs in the clearance of infection. β_2-Microglobulin-deficient mice have delayed viral clearance and increased mortality after a virulent influenza A virus infection (1). However, these mice can survive infection with a less virulent influenza A virus and can eliminate virus from the respiratory tract, whereas infection of nude mice or mice treated with antibodies to both CD4 and CD8 leads to death (14). These results suggest that $CD4^+$ CTLs may play a role in viral clearance that is detectable in the absence of a $CD8^+$ CTL response.

It is assumed that healthy adults may have had more than one exposure to influenza A virus because primary influenza A virus infections occur in early childhood (15). These primary natural infections in a nonimmune host result in extensive virus replication and would be expected to stimulate the influenza A virus-specific precursor CTL repertoire. Most of the CTLs we isolated recognize epitopes on highly conserved proteins and should be boosted by subsequent influenza A virus infections.

It is interesting that several novel T-cell epitopes could be defined by using PBMC of three donors. For example, cell line 10-1C4 of donor 1 recognizes NP aa 174 to 184, line 3E5 of donor 2 is specific to NP aa 256 to 266, line 77 of donor 3 recognizes NS1 aa 34 to 42, and CTL line 124 of donor 3 recognizes M2 aa 7 to 15. The broad CTL repertoire we have described may benefit humans, who are the natural host for these highly antigenically variable viruses. In addition to providing new information on the broad human repertoire of memory CTLs to influenza A viruses, the results suggest that many of the influenza virus structural and nonstructural proteins contain epitopes that may be useful to consider in vaccine development. It would be desirable to augment cross-reactive memory CTLs to provide a second line of defense against influenza disease, especially where major antigenic variation occurs at the antibody binding sites in the HA (23). These results suggest that humans have a broad repertoire of CTLs in response to influenza A virus.

ACKNOWLEDGMENTS

This work was supported partially by the NIAID, National Institutes of Health (AI 07349), and by Pasteur Merieux Connaught, Toronto, Ontario, Canada.

We thank Jack Bennink and Laurence Eisenlohr for providing the segmented NP recombinant vaccinia virus constructs, Bernard Moss for providing the other recombinant vaccinia virus constructs containing influenza A virus genes, and Alan Rothman for critically reading the manuscript. We thank Arthur Pedzcak, Pasteur Merieux Connaught, for providing NP, NS1, and M2 peptides.

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