The Journal of Immunology

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J Immunol 1999; 162:7578-7583; ;
http://www.jimmunol.org/content/162/12/7578

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Human CD8$^+$ and CD4$^+$ T Lymphocyte Memory to Influenza A Viruses of Swine and Avian Species

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Recently, an avian influenza A virus (A/Hong Kong/156/97, H5N1) was isolated from a young child who had a fatal influenza illness. All eight RNA segments were of avian origin. The H5 hemagglutinin is not recognized by neutralizing Abs present in humans as a result of infection with the human H1, H2, or H3 subtypes of influenza A viruses. Subsequently, five other deaths and several more human infections in Hong Kong were associated with this avian-derived virus. We investigated whether influenza A-specific human CD8$^+$ and CD4$^+$ T lymphocytes would recognize epitopes on influenza A virus strains derived from swine or avian species, including the 1997 H5N1 Hong Kong virus strains. Our results demonstrate that adults living in an urban area of the U.S. possess influenza A cross-serotype reactive CD8$^+$ and CD4$^+$ CTL that recognize multiple epitopes on influenza A viruses of other species. Bulk culture cytotoxicity was demonstrated against avian and human influenza A viruses. Enzyme-linked immunospot assays detected precursor CTL specific for both human CTL epitopes and the corresponding A/HK/97 viral sequences. We hypothesize that these cross-reactive CTL might provide partial protection to humans against novel influenza A virus strains introduced into humans from other species. ***The Journal of Immunology*****, 1999, 162: 7578–7583.**

Influenza A virus infections cause morbidity and excess mortality on an annual basis (1, 2). This excess mortality is usually observed in the elderly and in individuals with chronic illness. Older children and adults have generally had previous infections with influenza A viruses, but mutations at the major Ab combining sites allow reinfections to occur (3). These point mutations cause antigenic drift at the Ab combining sites on the external glycoproteins, hemagglutinin (HA)[3] and neuraminidase (NA). In addition, periodically a drastic Ag change occurs that may be due to a reassortment of genes from a human virus strain with genes from a nonhuman virus; this is called antigenic shift. The only times that these events have been documented were in 1957 and 1968, and marked increases in mortality and morbidity were noted (1). The most severe pandemic of influenza A virus that has occurred in modern times was the worldwide pandemic of 1918–1920 when over 20,000,000 deaths occurred (4). The virus that caused the 1918 pandemic has recently been partially characterized as having swine-like H1N1 sequences (5), and serologic studies of individuals who were alive during that pandemic also strongly suggest that the virus had a swine-like HA Ag (6).

Influenza virus-specific CTL have been shown in murine studies to limit influenza A virus replication and to protect against lethal influenza A virus challenge (7–12). CTL have been found in the lungs of influenza A virus-infected mice (8), and recovery from influenza infection has correlated with clearance by CD8$^+$ CTL (9). Virus-specific CTL clones that recognize epitopes on the nucleoprotein (NP), HA, nonstructural 1 (NS1) proteins, and influenza virus-stimulated immune splenocytes adoptively transferred into naive recipients reduced pulmonary virus titers after influenza virus challenge (7, 10–12). Our laboratory has shown that active immunization with an HA fusion protein that induced HA-specific CTL but not neutralizing Ab caused a reduction in peak lung virus titers after virus challenge and protection against a lethal challenge dose (13). Lack of CD8$^+$ CTL delays viral clearance and increases mortality after infection with a virulent strain of influenza virus (14). CD4$^+$ virus-specific T cells may help compensate for the absence of CD8$^+$ CTL because the virus can be cleared in CD8$^+$ CTL-deficient mice; however, mice lacking both CD4$^+$ and CD8$^+$ CTL do not clear virus or survive (15). Therefore, CTL seem to be important in both restricting influenza A virus replication and reducing disease severity.

In 1997, an H5N1 influenza A virus was isolated from a 3-year-old boy in Hong Kong. This child did not survive the infection, and his was the first of six deaths due to H5N1 influenza A virus infections (16). The isolated virus was found to be similar to avian H5N1 viruses (17, 18). We speculated that most of the older children and adults living in Hong Kong and elsewhere in the world in 1997 would possess influenza A virus cross-reactive memory T cells and that some of these clones would recognize epitopes on the H5N1 avian-derived virus strains that caused human illness. There have been no reports of the ability of human influenza A virus-specific CTL to recognize epitopes on nonhuman viruses. We have developed and characterized a panel of human CD8$^+$ and CD4$^+$ CTL lines from several donors residing in a city in the U.S. (19). These T cell lines were found to be either subtype specific or cross-reactive to the H1N1, H2N2, and H3N2 subtypes of human influenza A viruses (19). We determined whether these human CD8$^+$ and CD4$^+$ CTL lines would recognize epitopes on autologous cells infected with influenza A virus strains of swine and avian species including the H5N1 virus strains recently isolated from patients in Hong Kong. In addition, we tested bulk culture responses of these donors to determine whether CTL cross-reactivity could be detected at the population level and quantitated the precursor frequency of CTL specific for epitopes on the H5N1 virus.

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Received for publication February 8, 1999. Accepted for publication April 5, 1999.

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[2] Abbreviations used in this paper: HA, hemagglutinin; NA, neuraminidase; NP, nucleoprotein; NS1, nonstructural 1; M1, matrix 1; M2, matrix 2; B-LCL, B cell lymphoblastoid cell lines; ELISPOT, enzyme-linked immunospot.

0022-1767/99/$02.00

Materials and Methods

Viruses

Influenza A viruses, A/Puerto Rico/8/34 (H1N1) and A/Japan/305/57 (H2N2), were kindly provided from the Division of Virology (Bureau of Biologics, Food and Drug Administration, Bethesda, MD). A/Hong Kong/156/97 (H5N1) and A/Hong Kong/483/97 (H5N1) were kindly provided by Nancy Cox, World Health Organization Influenza Reference Laboratory, at the Centers for Disease Control and Prevention (Atlanta, GA). A/Duck/Pennsylvania/10218/84 (H5N2), A/Duck/Alberta/35/76 (H1N1), and A/New Jersey/8/76 (Hsw1N1) were provided by the American Type Culture Collection (Rockville, MD). Influenza A viruses were propagated in 10-day-old, embryonated chicken eggs. Infected allantoic fluids were harvested 2 days after infection with A/PR/8/34 or A/Japan/305/57 and 1 day after infection with A/HK/156/97 and A/HK/483/97, aliquoted, and stored at −80°C until use. Studies with the A/HK/97 virus strains were performed under biosafety laboratory level 3 conditions.

Human PBMC

PBMC specimens were obtained from normal, healthy, Caucasian American donors. PBMC were purified by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation (20). Cells were resuspended at 2×10^7/ml in RPMI 1640 with 20% FBS (Sigma Immunochemicals, St. Louis, MO) and 10% DMSO and cryopreserved until use. The HLA alleles of donor 1 were A2.1, A11, B18, B27, Cw1, Cw7, DR1, DQw1, DQw3, DRw52, DRw53; those of donor 2 were A2, A24, B7, B62, Cw3, DP2, DR1, DR2, DQw5, DQw6; and those of donor 3 were A1, B8, B44, Cw5, DR2, DR3, DQw1, DQw2, 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 (Life Technologies, Grand Island, NY) containing 10% human AB serum (NABI, Boca Raton, FL), penicillin-streptomycin, glutamine, and HEPES in a 70-ml Falcon flask (Becton Dickinson, Franklin Lakes, NJ). Stimulators were infected with the influenza A virus strain A/PR/8/34 (H1N1) at a multiplicity of infection of 15 for 1.5 h at 37°C in 1 ml PBS containing 0.1% BSA, and were then added to responders in a flask at a stimulator: responder ratio of 1:10. On day 7 of culture, cells were restimulated with γ-irradiated (3,000 rad) autologous PBMC infected with A/PR/8/34 at a multiplicity of infection of 15 for 1.5 h in 1 ml PBS containing 0.1% BSA, added at a stimulator:responder ratio of 1:10 in fresh medium containing 10% human AB serum and 20 U IL-2 (Collaborative Biomedical Products, Bedford, MA). Restimulated cells were assayed for cytolytic activity 7 days later.

CTL cell lines

Influenza-specific CTL clones were derived by methods that have been previously described (19, 21). PBMC that had been stimulated in bulk culture for 7 or 14 days were collected and plated at a concentration of 3, 10, or 30 cells/well in 96-well round bottom microtiter plates in 100 μl AIM-V medium containing 10% FBS, 20 U IL-2, 1:1000 dilution of the anti-CD3 mAb (12F6) kindly provided by Johnson Wong, and 10^5 γ-irradiated allogeneic PBMC/well. On day 7, 50 μl fresh medium with 10% FBS (Sigma) and IL-2 were added, and on day 14 fresh medium with 10^5 γ-irradiated allogeneic PBMC/well and 1:1000 dilution of anti-CD3 mAb were added. Growing cells were assayed for cytolytic activity on days 21 and 28. Cells from wells with influenza A-specific cytolytic activity were expanded to 48-well plates. Some T cell epitopes have been previously defined by others: NP 383–391 (22), M1 58–66 (23), NS1 122–130 (24), and M1 17–31 (25). We have identified additional $CD8^+$ and $CD4^+$ human T cell epitopes (19).

Preparation of target cells

Autologous B cell lymphoblastoid cell lines (B-LCL) were established by culturing with EBV in 24-well plates as previously described (26). Autologous B-LCL were infected with influenza A viruses in 1 ml 10% FBS in RPMI 1640 for 12–16 h. These infected target cells were labeled with 0.25 mCi ^{51}Cr for 60 min at 37°C. After four washes, the target cells were counted and diluted to 1.5×10^4 cells/ml for use in the cytotoxicity assay.

Cytotoxicity assays

Cytotoxicity assays were performed in 96-well round bottom plates, as previously reported (27). Briefly, effector cells in 100 μl RPMI 1640 medium containing 10% FBS were added to 1.5×10^3 ^{51}Cr-labeled target cells in 100 μl at various E:T ratios. In CTL 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. Peptides were synthesized at the Core Protein Chemistry Facility directed by Dr. R. Carraway (University of Massachusetts Medical Center, Worcester, MA). In assays testing bulk cultures, 1.5×10^4 K562 cells/well were added to the effectors for 2 h prior and left in during the assay to reduce NK cell activity. Plates were centrifuged at $200 \times g$ for 5 min and incubated for 4–5 h at 37°C. Supernatant fluids were harvested using the supernatant collection system (Skatron Instruments, Sterling, VA), and ^{51}Cr content was measured in a gamma counter. Spontaneous release was <30% in all assays unless otherwise indicated. The percent specific ^{51}Cr release was calculated as [(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. Specific immune lysis was calculated by subtracting the percent specific lysis of peptide-pulsed targets from lysis of uninfected targets.

Single-cell enzyme-linked immunospot (ELISPOT) assay for IFN-γ-secreting cells

The ELISPOT assay was done as previously described (19, 28). Briefly, 96-well filtration plates (Millipore, Bedford, MA) were coated with mouse anti-human IFN-γ Ab (clone NIB42, PharMingen, San Diego, CA). Cryopreserved PBMC were thawed, washed, and added to the plates at 5×10^5/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 peptide/ml). The plates were washed and then incubated with biotinylated mouse anti-human IFN-γ Ab (clone 4S.B3, PharMingen). Spots were developed using fresh substrate buffer (0.3 mg/ml of 3-amino-9-ethylcarbazole and 0.015% H_2O_2 in 0.1 M sodium acetate, pH 5). The precursor frequency of peptide-specific CTL was calculated based on the number of spots counted of the number of cells added to the wells.

Results

Human T cell line recognition of swine and avian influenza A virus infected targets

The only nonhuman influenza A virus we had available to begin these experiments was the influenza A/NJ/76 (Hsw1N1) virus, which was a swine-like influenza virus strain isolated from a young soldier who died of influenza in Fort Dix, NJ, in 1976 (3). The human $CD8^+$ or $CD4^+$ CTL lines previously established by stimulation with influenza A/PR/8/34 (H1N1) virus (19) were tested for recognition of epitopes on autologous target cells infected with the A/NJ/76 (Hsw1N1) virus. The results shown in Table I (Expt. A) indicate that about one-half of the $CD8^+$ human influenza A-specific, subtype cross-reactive CTL lines recognize and kill target cells infected with the swine-like A/NJ/76 virus at levels similar to those for lysis of target cells infected with the human A/PR/8/34 (H1N1) virus.

Avian virus strains that contained either the avian H5 HA (A/Duck/Pennsylvania/10218/84 [H5N2]) or the avian N1 NA (A/Duck/Alberta/35/76 ([H1N1)) similar to the H5 and N1 proteins of the recently isolated Hong Kong H5N1 viruses were then tested. The results in Table I (Expt. B) demonstrate that several of the human serotype cross-reactive CTL lines recognized autologous target cells infected with these avian viruses to a degree similar to that for the target cells infected with the human A/PR/8/34 (H1N1) strain. Certain $CD8^+$ and $CD4^+$ T cell lines that recognized epitopes only on H1 or on H1 and H2 subtype viruses did not recognize the avian virus-infected cells. A HLA-B27-restricted $CD8^+$ CTL line (1-1) from donor 1, which is known to recognize amino acids (aa) 383–391 on NP of the human subtypes H1, H2, and H3, recognized target cells infected with the avian 1976 H1N1 strain but did not recognize as well targets infected with the 1984 H5N2 avian virus. Similar results were obtained using another $CD8^+$ CTL line (10-2C2) which is HLA-A2.1 restricted and recognized aa 122–130 on the NS1 protein. However, another HLA-A2.1-restricted line (1-7-K) specific for an epitope on the matrix 1

Table I. *Recognition of swine and avian influenza A virus-infected target cells by human T cell lines*

						% Specific ^{51}Cr Release of Target Cells Infected with[c]					
						Expt. A		Expt. B		Expt. C	
Cell Line[a]	CD4/CD8	Protein	Epitopes[b] (amino acids)	Subtype Specificity	HLA Restriction	A/PR/8/34 (H1N1)	A/NJ/1/76 (Hsw1N1)	A/Duck/Pen/84 (H5N2)	A/Duck/Alb/76 (AvH1N1)	A/HK/156/97 (H5N1)	A/HK/483/97 (H5N1)
Donor 1											
1-1	CD8	NP	383–391	H1, H2, H3	B27	17.0	13.2	8.0	26.5	48.5	20.4
1-7-K	CD8	M1	58–66	H1, H2, H3	A2.1	45.6	31.4	43.5	42.5	64.9	57.9
10-2C2	CD8	NS1	122–130	H1, H2, H3	A2.1	20.0	15.1	−9.9	18.7	4.6	−1.3
10-1C4	CD8	NP	174–184	H1, H2, H3	B27	10.1	5.4	−4.0	3.4	13.7	6.3
10-1G5	CD8	HA	—	H1, H2	B18	26.8	7.2	3.3	10.0	41.9	18.8
10-1B7	CD8	PB2	—	H1, H2, H3	B27	43.8	8.7	14.3	30.7	61.3	55.4
1-2F8	CD8	PB1	—	H1, H2, H3	B27	21.1	10.1	12.4	13.0	41.9	15.7
1-3	CD4	M1	17–31	H1, H2, H3	DR1	65.6	65.2	19.1	20.3	33.1	2.6
10D9-d10	CD4	NA	—	H1	DR1	37.5	−3.1	−10.5	−1.8	7.9	−2.5
Donor 2											
3G11	CD8	NP	315–496	H1, H2, H3	B62	26.7	41.7	73.6	65.5	79.7	84.0
3E5	CD4	NP	254–262	H1, H2, H3	—	76.9	77.9	80.8	69.4	82.2	75.1
3E9	CD4	NA	—	H1	—	14.1	3.2	−1.2	53.7	14.4	9.7
10E7	CD4	M1	—	H1, H2, H3	—	24.5	21.8	50.0	49.8	83.3	79.6
Donor 3											
124	CD8	M2	7–15	H1, H2	B44	26.8	2.6	−0.8	1.5	−2.8	−0.7
77	CD4	NS1	34–42	H1, H2, H3	DR3	79.1	71.3	3.8	73.8	68.5	65.4

[a] Each donor was tested separately in three experiments.
[b] —, HLA restriction or epitope not yet defined.
[c] These results were obtained using PBMC of American donors; although the frequency of HLA alleles varies considerably between distinct population groups, some, e.g., HLA A2, represent ~30% of both North American Caucasian and Southern Han, the dominant tribe in southern China (31). E:T ratio was 10:1–20:1 and uninfected targets were used as a negative control with lysis <1% by all of the T cell lines.

(M1) protein recognized target cells infected with either of these avian viruses to a similar degree as A/PR/8/34-infected target cells.

The 1997 H5N1 Hong Kong virus strains were subsequently tested for recognition as virus-infected autologous target cells by these human T cell lines (Table I, Expt. C). Several of the donor 1 $CD8^+$ cell lines recognized the H5N1 virus-infected target cells, e.g., cell line 1-1 which is HLA-B27 restricted and recognizes aa 383–391 on NP, and cell line 1-7-K which is HLA-A2.1 restricted and recognizes aa 58–66 on the M1 protein. Other $CD8^+$ CTL lines recognized target cells infected with H1N1 virus but not targets infected with the Hong Kong H5N1 strains, e.g., cell line 10-2C2 which is HLA-A2.1 restricted and recognizes aa 122–130 on NS1. Cell lines 1-2F8 and 10-1B7, both HLA-B27 restricted, recognize epitopes on the polymerase (PB1 and PB2) proteins, and these cell lines recognized target cells infected with the 1997 Hong Kong viruses to a similar degree as the earlier human H1 virus. The subtype-specific $CD4^+$ CTL line 10D9-d10 which is HLA-DR1 restricted and recognizes an epitope on NA only recognized target cells infected with A/PR/8/34 virus (H1N1).

Similar results were obtained using cell lines derived from donor 2. Entirely cross-reactive killing of target cells infected with the H1 or the H5N1 Hong Kong strains was seen with one $CD8^+$ CTL line, 3G11, which is HLA-B62 restricted and recognizes an epitope located within aa 315–496 on NP. Two $CD4^+$ CTL lines that recognize epitopes on NP (3E5) and M1 (10E7) were also cross-reactive to the Hong Kong avian virus-infected target cells. However, an NA-specific $CD4^+$ line 3E9 recognizes only H1N1 virus-infected target cells. One cell line from the third donor exhibited a very cross-reactive pattern of lysis, while a second cell line exhibited a specific pattern of lysis. The $CD4^+$ CTL line 77 restricted by HLA-DR3 recognizes an epitope on aa 34–42 on the NS1 protein, and lysed target cells infected with the human H1 virus and the 1997 Hong Kong virus strains. On the other hand, the HLA-B44-restricted $CD8^+$ CTL line 124, which recognizes aa 7–15 on the matrix 2 (M2) protein, killed target cells infected with human H1N1 subtype virus but did not recognize target cells infected with either of the 1997 Hong Kong viruses.

Bulk culture PBMC lysis of avian, swine, and human-derived influenza A virus-infected targets

Bulk cultures of human A/PR/8/34 virus-stimulated PBMC effector cells were tested for recognition of autologous B-LCL infected with human H1N1 or H2N2 viruses, older avian viruses (H5N2 and H1N1), the recent avian-derived viruses isolated from humans in 1997 in Hong Kong (H5N1), or the swine-like 1976 virus (Hsw1N1). The results in Fig. 1 demonstrate convincing killing by cells in these bulk cultures of target cells infected with the recent Hong Kong viruses to a degree similar to that of target cells infected with the older human H1N1 and H2N2 virus strains. Killing was also observed of target cells infected with the older avian virus strains and the Hsw1N1 strain. This experiment demonstrates that humans have memory T lymphocytes that recognize epitopes on avian- and swine-derived viruses, including the recent Hong Kong-derived strains, in a highly cross-reactive fashion.

Human T cell line recognition of mutated peptides from the A/HK/156/97 virus strains

Next, the effect of mutations in the A/HK/156/97 (H5N2) virus sequences at peptide epitope sites recognized on the A/PR/8/34 (H1N1) sequence was examined. The results in Table II show a variety of mutations and recognition patterns. Cell line 10-2C2, a $CD8^+$ CTL that recognizes aa 122–130 on the NS1 protein of H1N1, H2N2, and H3N2 viruses, does not recognize target cells pulsed with the A/HK/156/97 peptide due to a single N → D mutation at aa 127. The $CD4^+$ cell line 3E5, which recognizes aa 254–264 on the NP of A/PR/8/34, also recognized targets pulsed with the A/HK/156/97 peptide despite a T → I mutation at aa 258. A $CD8^+$ CTL line 124, which recognizes an epitope on M2 aa 7–15 on the H1N1 subtype virus, does not recognize the H5N1 peptide which has changes at aa 10, 11, and 14. These results

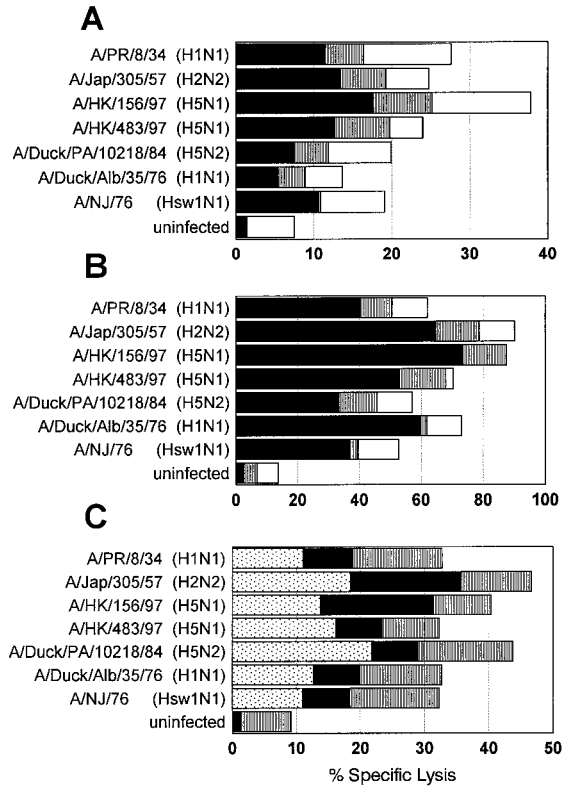

FIGURE 1. Bulk culture CTL recognize avian-, swine-, and human virus-infected targets. Bulk culture chromium release assays of donor 1 (*A*), donor 2 (*B*), and donor 3 (*C*) PBMC after stimulation with A/PR/8/34 (H1N1) on days 0 and 7. Specific ^{51}Cr release was measured on autologous B-LCL target cells infected with indicated virus strains at E:T ratios of 100:1 (□), 50:1 (▨), 25:1 (■), and 12.5:1 (▩).

illustrate that some epitopes of avian viruses can still be recognized by human CTL, while others cannot.

Precursor CTL detection by ELISPOT

Table III demonstrates the precursor frequency of CTL detected in PBMC to two different epitopes of the human H1N1 and Hong Kong H5N1 viruses determined using an ELISPOT assay to quantitate the number of IFN-γ-producing cells. In donor 1, stimulation of PBMC with the peptide based on NP aa 174–184 of A/PR/8/34 (H1N1) had a precursor CTL of 1/4348. Stimulation with the NP 174–184 peptide based on the A/Hong Kong/156/97 strain with a V → I change at aa 183 produced a similar number of IFN-γ-secreting cells. On the other hand, the E → Q change at aa 23 in the M1 protein on the avian Hong Kong 483 strain resulted in the loss of detectable precursors in this donor when used as a peptide to stimulate PBMC. Thus the quantitative detection of individual peptide-specific T cells showed either a similar frequency of precursor CTL or an absence of precursor CTL, depending on the specific epitope and the mutations present in the recently isolated Hong Kong viruses.

A summary of the effect of sequence conservation and mutation at CTL epitopes in the human isolates of avian-derived virus (A/HK/156/97 and A/HK/483/97 (H5N1)) is presented in Table IV. Sequence data are available for comparison of seven human CTL epitopes. Three epitopes are entirely conserved and are cross-reactive. One CD4$^+$ CTL epitope (M1 protein aa 17–31 restricted by DR1) is conserved on A/HK/156/97, but not on A/HK/483/97. The E → Q change at aa 23 is not recognized. There are three other epitopes that are not conserved; a B27-restricted clone recognizes a mutation on NP aa 183 (V → I). The other two epitopes with mutations are not recognized: an HLA A2.1-restricted T cell epitope aa 122–130 has a N → D change at aa 127 and a B44-restricted epitope on M2 aa 7–15 has three aa changes. Therefore, there is cross-reactive recognition by the human CTL clones of the avian virus strains that are conserved or have conservative aa changes in CTL epitopes, but mutation at several other epitopes results in a lack of recognition.

Discussion

These results demonstrate that adults living in an urban area of the U.S. have influenza A-specific CD8$^+$ and CD4$^+$ memory T lymphocytes that recognize epitopes on influenza A virus strains derived from swine and avian species including the H5N1 viruses that were isolated from patients infected in Hong Kong in 1997. This is the first report of the recognition of human target cells infected with influenza A viruses of nonhuman species by autologous CD8$^+$ or CD4$^+$ CTL induced by prior human influenza A virus infection. The results clearly show that adult humans with no known exposure to these nonhuman species possess high levels of memory CTL which are readily detected in bulk culture assays following stimulation with a human influenza A virus (Fig. 1). The recognition of autologous cells infected with the recent Hong Kong H5N1 isolates or the older avian and swine-derived viruses was convincing and was similar to the level of lysis of autologous cells infected with human influenza A viruses. These bulk culture results were in accord with the specific CTL lysis of target cells infected with the swine and avian-derived virus strains and the recent H5N1 isolates from Hong Kong by a panel of human CD8$^+$ and CD4$^+$ T cell lines, the majority of which are cross-reactive among the human H1, H2, and H3 subtypes.

The cross-reactive recognition of epitopes by these CD8$^+$ and CD4$^+$ T cell clones fits well with available sequence data. Clones that recognized defined epitopes with no or minimal conserved mutations recognized target cells infected with the swine-derived

Table II. *Effect of mutations on the HK/156/97 virus on CTL recognition by human CD8$^+$ and CD4$^+$ CTL lines to H1N1 epitopes*

Donor	Cell Line	CD8/CD4	Protein Specificity	Epitope	Virus Strain	% Specific Lysis of Peptide Pulsed Targets 2.5 μg/ml	0.25 μg/ml	0.025 μg/ml	Peptide Sequence[a]
1	10-2C2	CD8	NS1	122–130	A/Udorn/74	77.2	79.1	69.6	A I M D K N I M L
					A/HK/156/97	−5.1	−7.1	−5.6	* * * * * D * I *
2	3E5	CD4	NP	254–264	A/PR/8/34	16.7	2.5	0.5	E D L T F L A R S A L
					A/HK/156/97	19.1	1.3	0.3	* * * I * * * * * * *
3	124	CD8	M2	7–15	A/PR/8/34	75	65.5	15.1	V E T P I R N E W
					A/HK/156/97	−1.9	−2.1	−1.6	* * * L T * * G *

[a] *, Same amino acid as above.

Table III. *Precursor frequency analysis of mutations in HK/156/97 and HK/483/97 using ELISPOT assay*

Donor	Protein Specificity	Epitope	Virus Strain	Precursor CTL Frequency	Peptide Sequence
1	NP	174–184	A/PR/8/34	1/4,348	R R S G A A G A A V K
			A/HK/156/97	1/5,263	* * * * * * * * * I *
1	M1	17–31	A/PR/8/34	1/50,000	S G P L K A E I A Q R L E D V
			A/HK/483/97	<1/500,000	* * * * * * Q * * * * * * * *

*, Same amino acid as above.

Hsw1N1, the older avian virus strains H5N2 and H1N1, and the recent H5N1 isolates from Hong Kong similar to target cells infected with human H1N1 virus. For example, the conservative T → I change in the epitope recognized by the $CD4^+$ T cell line 3E5 at position 4 of the A/HK/156/97 strain did not abrogate recognition. A minority of these human T cell lines failed to recognize other strains when certain nonconservative mutations occurred. This is seen with the epitope recognized by $CD8^+$ cell line 10-2C2, where a N is mutated to a D at position 6 (aa 126 on the NS1 protein) in the A/HK/156/97 strain. Thus, having an acidic amino acid at this position that is usually oriented toward the cleft top (29) abrogates recognition by this cell line. Other avian viruses such as A/Duck/Ukraine/1/63 also have this mutation at amino acid 127 of NS1 (24).

The level of influenza A virus peptide-specific precursor CTL has been shown to correlate with the number of IFN-γ-producing cells in PBMC after a brief exposure to peptide (19, 28). The estimation of precursor frequency in PBMC using an ELISPOT assay was in accord with the results of the CTL assays performed with bulk cultures and with the T cell lines. CTL lines, which killed target cells infected with human subtypes and viruses derived from avian species in a cross-reactive fashion, were detectable as single cells in PBMC at similar precursor frequencies following stimulation with the human H1N1 peptide or the corresponding peptide from the H5N1 Hong Kong virus. This was seen using peptides based on the NP epitope with amino acids 174–184 which has a conservative V → I change in the A/HK/156/97 strain and a precursor frequency similar to that detected with the A/PR/8/34 sequence. On the other hand, peptides of the H5N1 virus with mutations in the epitope that resulted in no lysis of virus-infected or peptide-pulsed target cells had an undetectable precursor frequency following stimulation with the H5N1 peptide. For example, the E → Q mutation at position 7 in the M1 epitope with aa 17–31 abrogated IFN-γ secretion. The precursor frequency derived using the human H1N1 viral peptide stimulation was much higher than that of the A/HK/483/98 peptide. Therefore, replacing the acidic glutamic acid residue with the neutral glutamine residue at position 7 on this epitope decreased IFN-γ secretion by the PBMC of donor 1. Another group found that changing this amino acid to an asparagine, along with a Y at position 3 and a K at position 10 abrogates proliferation of T cell clones (30).

What are the effects of these preexisting human influenza A virus cross-reactive memory T cells when a new nonhuman influenza A virus is introduced into humans, as occurred in Hong Kong in 1997 (3) and might have occurred in 1918? There was relatively little influenza A virus activity noted in the two decades preceding the 1918 pandemic (4). An epidemic occurred in 1890 which appears to have been caused by a virus with an H3-like HA based on

Table IV. *Human CTL recognition of sequences in avian (A/Hong Kong/97 [H5N1])-derived strains*

Protein Specificity	MHC	Epitope	Strain Sequence[a]	Virus Strain	Relative SIL[b]	Accession Number
NS1	A2.1	122–130	A I M D K N I M L	A/Udorn/74	+	A04088
			* * * * * * * I *	A/PR/8/34	+	J02150
			* * * * * D * I *	A/HK/156/97	−	AF036360
			* * * * * D * I *	A/HK/483/97	−	—[c]
M2	B44	7–15	V E T P I R N E W	A/PR/8/34	+	M10642
			* * * L T * * G *	A/CHICK/PENN/1370/83	−	(32)
			* * * L T * * G *	A/HK/156/97	−	AF036358
			* * * L T * * G *	A/HK/483/97	−	—[c]
NP	B27	383–391	S R Y W A I R T R	A/PR/8/34	+	J02147
			* * * * * * * * *	A/NJ/8/76	+	M63754
			* * * * * * * * *	A/HK/156/97	+	AF036359
			* * * * * * * * *	A/HK/483/97	+	—[c]
M1	A2.1	58–66	G I L G F V F T L	A/PR/8/34	+	M10642
			* * * * * * * * *	A/HK/156/97	+	AF036358
			* * * * * * * * *	A/HK/483/97	+	—[c]
NP	B27	174–184	R R S G A A G A A V K	A/PR/8/34	+	J02147
			* * * * * * * * * I *	A/HK/156/97	+	AF036359
			* * * * * * * * * I *	A/HK/483/97	+	—[c]
NS1	DR3	34–42	D R L R R D Q K S	A/PR/8/34	+	J02150
			* * * * * * * * *	A/HK/156/97	+	AF036360
			* * * * * * * * *	A/HK/483/97	+	—[c]
M1	DR1	17–31	S G P L K A E I A Q R L E D V	A/PR/8/34	+	M10642
			* * * * * * * * * * * * * *	A/HK/156/97	+	AF036358
			* * * * * * Q * * * * * * * *	A/HK/483/97	−	—[c]

[a] Sequences obtained from GenBank database. *, Same amino acid as above.

[b] Relative specific immune lysis (SIL) = % specific immune lysis by human CTL clones of human influenza strain epitope peptides. Lysis of avian peptide sequence is indicated as positive (+) with specific lysis >15% or not detected (−).

[c] K. Subbarao, unpublished observations.

serological evidence of H3-specific Abs found before 1968 in the sera of individuals born before 1890 (6). The reasons for the tremendous mortality during the 1918 pandemic are unknown. Young adults, especially the age group of 25–35 years, had very high mortality rates in 1918 which is unusual in influenza; deaths usually occur in the very young and in older individuals (4). Thus, a ∨∨-shaped curve in age-related mortality with the peak deaths between the ages of 25–35 was described instead of the usual ∪-shaped curve with deaths peaking only in the very young and old (4). In addition to unusual virulence of the 1918 virus, responses, including cross-reactive influenza-specific T cell responses, may have been low or absent in some of the young adult cohort because there was relatively little influenza A activity noted during the two decades immediately before 1918 (4).

The impact of influenza A cross-reactive memory CTL on the morbidity and mortality associated with influenza virus epidemics and the pandemics of 1918, 1957, and 1968 is unknown. In 1957, most adults presumably had cross-reactive CTL memory to epitopes on the H1N1 viruses which circulated widely before the "Asian" pandemic (H2N2) of 1957. Similarly, when the H3N2 "Hong Kong" virus emerged in 1968, adults and older children would be expected to have had cross-reactive memory CTL as a result of infection with the prior H2N2 virus strains, and adults would also have been exposed to the earlier H1N1 strains. The pandemic of 1957 caused more deaths than that of 1968, but fortunately this was only a fraction of the deaths that were observed in 1918. Although it is clear that cross-reactive memory T cells did not prevent infections and excess mortality in 1957 and 1968, these memory T lymphocytes may have contributed a degree of partial protection by limiting the degree of viral replication, based on experimental studies of CTL in mouse models (7, 10–12).

Overall, these results suggest that adults living in urban areas have $CD8^+$ and $CD4^+$ memory CTL as a result of prior infection with human influenza A viruses, and these CTL are in large part cross-reactive to epitopes on influenza A virus strains derived from swine and avian species. Although we detected mutations at several human CTL epitopes, some mutant virus sequences were still recognized by these clones. Most of the human CTL clones were able to recognize entirely conserved or mutated viruses, consistent with the results of our bulk culture experiments. There is considerable conservation among the genes encoding the internal and nonstructural proteins of influenza A virus, which is consistent with our results. The presence of these memory T lymphocytes may play a role in helping to restrict virus replication, thereby decreasing morbidity and mortality to a degree. Our results support the hypothesis that cross-reactive T cells will be activated in humans by infection with a novel influenza A virus derived from another species, and the activation of these memory CTL by infection may result in reduced replication of the new infecting virus.

Acknowledgments

We thank Nancy Cox and Kanta Subbarao for providing the 1997 Hong Kong virus strains and the sequence information of the A/Hong Kong/483/97 strain, Alan Rothman for reviewing the manuscript, and Stephen Schoenbaum for information regarding the 1918 epidemic.

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