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# Human CD4$^+$ T-Cell Repertoire of Responses to Influenza A Virus Hemagglutinin after Recent Natural Infection

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**The human CD4$^+$ T-cell repertoire of responses to hemagglutinin (HA) of influenza virus A/Beijing/32/92 was examined 3 to 6 months after natural infection by using a panel of 16-mer peptides overlapping by 11 residues. Short-term CD4$^+$ T-cell lines were derived by using full-length HAs of virus A/Beijing/32/92 from 12 unrelated, major histocompatibility complex (MHC) class I and II haplotyped adults with a history of influenza in November and December 1993 and from 6 adults with no history of influenza during the preceding 4 years but who responded to HA. In contrast to recent murine studies, the human CD4$^+$ T-cell repertoire of responses was dominated by the recognition of highly conserved epitopes. The HA2 subunit, widely regarded as nonimmunogenic, induced strong responses in every donor. This resulted in functional cross-reactivity among influenza A viruses. Our study included one pair of unrelated donors expressing identical HLA DRB1* and DQB1* alleles and two pairs of donors sharing low-resolution MHC class II types. These pairs responded to identical peptides; furthermore, clearly identifiable patterns of response were seen in donors sharing single class II haplotypes, irrespective of the presence of other alleles and exposure history. Two conserved regions which induced responses in 17 of 18 donors were identified (residues 295 to 328 and 407 to 442). Possible implications for cross-reactive T-cell vaccines are discussed.**

Influenza virus type A remains a major cause of human morbidity and mortality. Humoral immunity is hindered by antigenic shift and drift of the viral surface coat proteins (2). The major surface glycoprotein hemagglutinin (HA), which consists of two subunits, HA1 and HA2, produced by enzymatic cleavage of a precursor molecule, HA0, is responsible for virus-host cell fusion. The HA1 subunit contains both highly conserved and variable regions located at five main sites on the surface of the molecule (36); these are the targets of the host neutralizing antibodies. HA2 is conserved in structure among H3 influenza A viruses and has homology with influenza type A H1 and H2 viruses. CD4$^+$ T-cell help is required for the production of neutralizing antibody (1, 34), and HA is a major antigen for both the murine and the human CD4$^+$ T-cell responses to influenza virus (14, 16, 20, 24).

Murine studies have demonstrated that CD4$^+$ T cells recognize both conserved and variable regions of HA, depending on the experimental system used (reviewed in reference 37). Thomas and associates have shown, in a series of detailed experiments, that following nasal priming with live virus, strong CD4$^+$ T-cell responses to epitopes closely related to the highly variable, antibody-neutralizing sites of HA occur (3, 4, 10, 15, 24, 32), indicating that murine T- and B-cell epitopes can overlap (4).

Very few studies of the human CD4$^+$ T-cell response to influenza A virus HA have been undertaken, and the responses of CD4$^+$ T cells derived from only a single donor have been mapped in detail (19, 21); even this study employed an incomplete panel of peptides derived from only the HA1 sequence of a viral strain that had not circulated for 10 years. However, the indication from this study and two other studies using T-cell clones derived from single donors and panels of influenza viruses (14) or HAs (31) is that the human response may be directed, at least in part, towards conserved regions of HA.

In order to obtain detailed information about the human CD4$^+$ T-cell repertoire of responses after recent natural infection, we investigated the responses of CD4$^+$ T cells from a panel of 12 unrelated major histocompatibility complex (MHC) class I- and II-typed adult donors with a history of influenza virus infection during the winter of 1993 to 1994 (recent influenza virus donors). We employed HA and peptides derived from influenza virus A/Beijing/32/92, the main H3 influenza virus circulating at the time of our recent influenza virus donors' illnesses. We aimed to examine the balance of conserved and variable epitope recognition following natural infection, to examine the CD4$^+$ T-cell response to both the HA1 and the HA2 subunits, and to investigate the MHC genetics of peptide recognition. We decided to study the responses of CD4$^+$ T-cell lines rather than T-cell clones because the use of clones would have severely restricted the number of donors examined, would have led to the selection of a small number of dominant epitopes that were not necessarily representative of the CD4$^+$ T-cell repertoire, and might therefore have introduced bias in favor of conserved-epitope recognition. We employed a panel of CD4$^+$ T-cell lines selected with full-length HA for 2 to 3 weeks and examined their proliferative responses to HA from virus A/Beijing/32/92 and HA derived from the prototype H3 subtype virus A/Aichi/68. Epitope recognition was mapped by using a panel of 118 16-mer peptides, overlapping by 11 residues, spanning the entire sequence of A/Beijing/32/92 HA.

In order to compare the results obtained within 6 months after infection with longer-term CD4$^+$ T-cell memory, we also studied a panel of six unrelated adult volunteers who had been

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TABLE 1. Subject details[a]

| Donor | Age (yr) | Sex[b] | Haplotype | | Duration of illness (days) | Cell recovery[c] |
|---|---|---|---|---|---|---|
| | | | MHC class I | MHC class II | | |
| A | 35 | F | A2,68, B18,44, CW7,7/8V | DRB1*0101,11, DRB3*01/02/03, DQB1*0301,05 | 7 | 7.9 |
| B | 55 | F | A1, B18,27, CW1,7 | DRB1*0101,11, DRB3*01/02/03, DQB1*0301,05 | 14 | 4.5 |
| C | 35 | M | A2,3, B7,44, CW5,7 | DRB1*0401,15, DRB4*01, DRB5*01, DQB1*0301,0601/2 | 10 | 13.3 |
| D | 24 | F | A1,72, B7,37, CW6,7 | DRB1*0408,15, DRB4*01, DRB5*01, DQB1*0301,0601/2 | 10 | 4.7 |
| E | 25 | F | A2, B44/63, CW1,5 | DRB1*0101,0401, DRB4*01, DQB1*0301,05 | 10 | 4.5 |
| F | 21 | F | A2,3, B7,44, CW5,7 | "DRB1*03,04, DRB3*01/02/03, DRB4*01, DQB1*02"[d] | 14 | 4.4 |
| G | 40 | F | A2, B15,65, CW3,10 | DRB1*10,1302, DRB3*01/02/03, DQB1*05,0604-8 | 14 | 13.4 |
| H | 24 | F | A11,24, B52,75, CWND | DRB1*0403,12, DRB3*01/02/03, DRB4*01, DQB1*0301,0302 | 14 | 8.1 |
| I | 25 | M | A2, B14, CW5 | DRB1*0102,07, DRB4*01, DQB1*02,05 | 14 | 10.4 |
| J | 24 | M | A1,2, B8,52, CWND | DRB1*15,0301, DRB3*01/02/03, DQB1*02,0601/2 | 7 | 3.6 |
| K | 25 | M | A24, B55,62, CW9 | DRB1*1302, DRB3*01/02/03, DQB1*02,06 | 7 | 4.4 |
| L | 25 | F | A30,68, B41,51, CW(1501-3),(41-42) | DRB1*1301,1303/4, DRB3*01/02/03, DQB1*0301,603 | 5 | 7.0 |
| M | 38 | M | A2,3, B7,51, CW1,7 | DRB1*0101, DQB1*05 | 0 | 1.2 |
| N | 30 | M | A2,66, B27,51, CW1,7 | DRB1*0103,08, DQB1*0301,04 | 0 | 0.7 |
| O | 42 | M | A2,31, B51,60, CW4,10 | DRB1*0101,0402, DRB4*01, DQB1*0302,05 | 0 | 0.6 |
| P | 34 | M | A2,28, B7,17, CW6,7 | DRB1*15,07, DRB4*01, DRB5*01, DQB1*0303,601/2 | 0 | 0.6 |
| Q | 36 | F | A2,19, B14,18, CWND | DRB1*0101,12, DRB3*01/02/03, DQB1*0301,05 | 0 | 2.1 |
| R | 28 | M | ND[e] | DRB1*0101,07, DRB4*01, DQB1*02,05 | 0 | 1.5 |

[a] Subjects A to L had a history of influenza during November and December 1993; subjects M to R had no history of influenza during the preceding 4 years.
[b] F, female; M, male.
[c] Ratio of cells recovered at day 21 in culture to number of PBMC initially seeded, calculated as follows: (number of cells recovered on day 7/number of PBMC originally seeded) × (number recovered on day 14/number seeded on day 7) × (number recovered on day 21/number seeded on day 14).
[d] Subject F was class II typed serologically only, with the typing reported in molecular typing nomenclature for comparison.
[e] ND, not done.

found to make polyclonal responses to HA but who gave no history of influenza during the preceding 4 years (control donors).

## MATERIALS AND METHODS

**Subject selection.** Twelve unrelated healthy adult donors (ages, 21 to 55 years) with a history of influenza virus infection during November and December of 1993 were studied (recent influenza donors, subjects A to L) 3 to 6 months after infection. None had ever been vaccinated against influenza virus. A positive history was taken to be fever, headache, sore throat, myalgia, and severe lassitude lasting several days during a period when A/Beijing/32/92-like strains were circulating. Many of the subjects had extended periods of lassitude lasting several months and/or persistent cough. Three of the donors (D, G, and I) had been examined for responses to HA during the summer of 1993 and were found to be nonresponders.

An additional group of six healthy subjects (ages, 28 to 42 years), with no history of influenza-like illness during the previous 4 years but who made a strong polyclonal T-cell response to HA, were studied as a comparison (control donors, M to R). Subject details are summarized in Table 1.

**MHC typing.** MHC class I typing was performed by a combination of serological typing at the Tissue Typing Laboratory, St. Mary's Hospital, London, United Kingdom (33), and molecular typing using sequence-specific primers (PCR-SSP) at the Tissue Typing Laboratory, Churchill Hospital, Oxford, United Kingdom (9, 30). MHC class II typing was performed by molecular typing using PCR-SSP at the Tissue Typing Laboratory, Churchill Hospital (26), with the exception of one donor (F) who was typed serologically at St. Mary's Hospital. All donors' tissue types are presented in the format recommended in "Nomenclature for Factors of the HLA System, 1994" (6).

**Antigens.** X117 recombinant A/Beijing/32/92 HA was the generous gift of R. Brands, Solvay Duphar, Weesp, The Netherlands. The preparation was egg derived and contained the entire HA1 and HA2 subunits. The preparation was contaminated with a trace of nucleoprotein. HA from A/Aichi/68 was the generous gift of A. Hay and J. Skehel, National Institute for Medical Research, Mill Hill, London, United Kingdom, and was obtained by bromelain cleavage (7). A series of overlapping peptides were derived from the nucleotide sequence of the HA1 subunit of A/Beijing/32/92 and the HA2 subunit of the closely related virus A/Hong Kong/90, as sequence information for the HA2 subunit of A/Beijing/32/92 was unavailable at the time of peptide synthesis. Unpublished sequence information for both viruses was kindly supplied by N. Cox (Centers for Disease Control and Prevention, Atlanta, Ga.). Peptides were synthesized by using F-moc chemistry on a Multipeptide Synthesis block BT7400 (Cambridge Research Biochemicals, Wirral, United Kingdom) with Pepsyn KB resins by the method of Fairchild et al. (13). In brief, couplings were performed by using F-moc side chain protected pentafluorophenyl or oxobenzotriazine amino acid esters in the presence of 1-hydroxybenzotriazole. Full-length peptides were deprotected with trifluoroacetic acid-phenol-thioanisole-ethanedithiol in the ratio of 94:2:2:2 and cleaved with 0.4 M NaOH. The resulting solutions were neutralized with HCl to yield peptides in saline solution. The peptides were 16 residues long, overlapping normally by 11 amino acids. The composite primary amino acid sequence used to synthesize the peptides and individual peptide sequences are shown in Fig. 1, which also shows the residue changes between the HAs from A/Aichi/68 and A/Beijing/32/92 as an indication of the variable regions of H3 HA. The numbering of peptides used in this study is based on the precursor molecule HA0.

**Establishment of $CD4^+$ T-cell lines.** Venous blood was collected between 3 and 6 months after infection, and peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque density centrifugation. A total of $25 \times 10^6$ PBMC were cultured for 7 days in 24-well tissue culture plates at 37°C with 5% $CO_2$ with HA from A/Beijing/32/92 (0.1 μg/ml) at a density of $5 \times 10^6$ cells per well, in 2 ml of complete medium (RPMI 1640 supplemented with 2 mM L-glutamine, 100 IU of penicillin-streptomycin [Gibco, Life Technologies, Paisley, Scotland] per ml, and 5% screened, inactivated human $AB^+$ serum [Sigma, Poole, Dorset, England]). The remaining PBMC were stored in liquid $N_2$ and subsequently used as antigen-presenting cells (APCs).

At day 7, $4 \times 10^6$ T cells were used to analyze the response to HA and peptides as described below and the remaining T cells were restimulated, at a density of $1.0 \times 10^6$ per well, with equal numbers of irradiated (3,000 rads) autologous PBMC preincubated at 37°C in 5% $CO_2$ in complete medium for 1 h with HA from A/Beijing/32/92 (0.01 to 0.1 μg/ml). At 24 and 72 h, the lines were supplemented with a source of interleukin 2 (Lymphocult T; Biotest Folex, Frankfurt, Germany) (10%, vol/vol). The T-cell lines were maintained in culture for 3 to 4 weeks as described above, with the addition of fresh, irradiated autologous prepulsed PBMC every 7 days.

**Analysis of T-cell specificities by using overlapping peptides.** Autologous, irradiated (3,000 rads) PBMC ($4 \times 10^4$/well) were incubated with whole antigen (HA from A/Beijing/32/92 [0.01 to 0.1 μg/ml] or HA from A/Aichi/68 [1.0 μg/ml]) or peptide pools (five peptides each at a concentration of 10 μg/ml) for 1 h at 37°C in 5% $CO_2$ in complete medium and subsequently cultured with $4 \times 10^4$ responder T cells per well in round-bottommed 96-well plates. At 48 h, the cells were pulsed with [$^3$H]thymidine (1 μCi per well; Amersham International PLC, Amersham, United Kingdom) and harvested 16 h later. Proliferation, as correlated with [$^3$H]thymidine incorporation, was measured by liquid scintillation spectroscopy. Responses of at least five times the geometric mean of the background (T cells, autologous irradiated PBMC, and medium) were scored as positive. Mapping with individual peptides (10 μg/ml) was conducted at day 21 or 28 as described above.

**MHC class II restriction studies.** Monoclonal anti-HLA-DR (L243) (22), anti-HLA-DP (B7/21) (35), and anti-HLA-DQ (SPV-L3) (a gift from H. Spitz, DNAX, Palo Alto, Calif.) antibodies were isolated from hybridoma culture

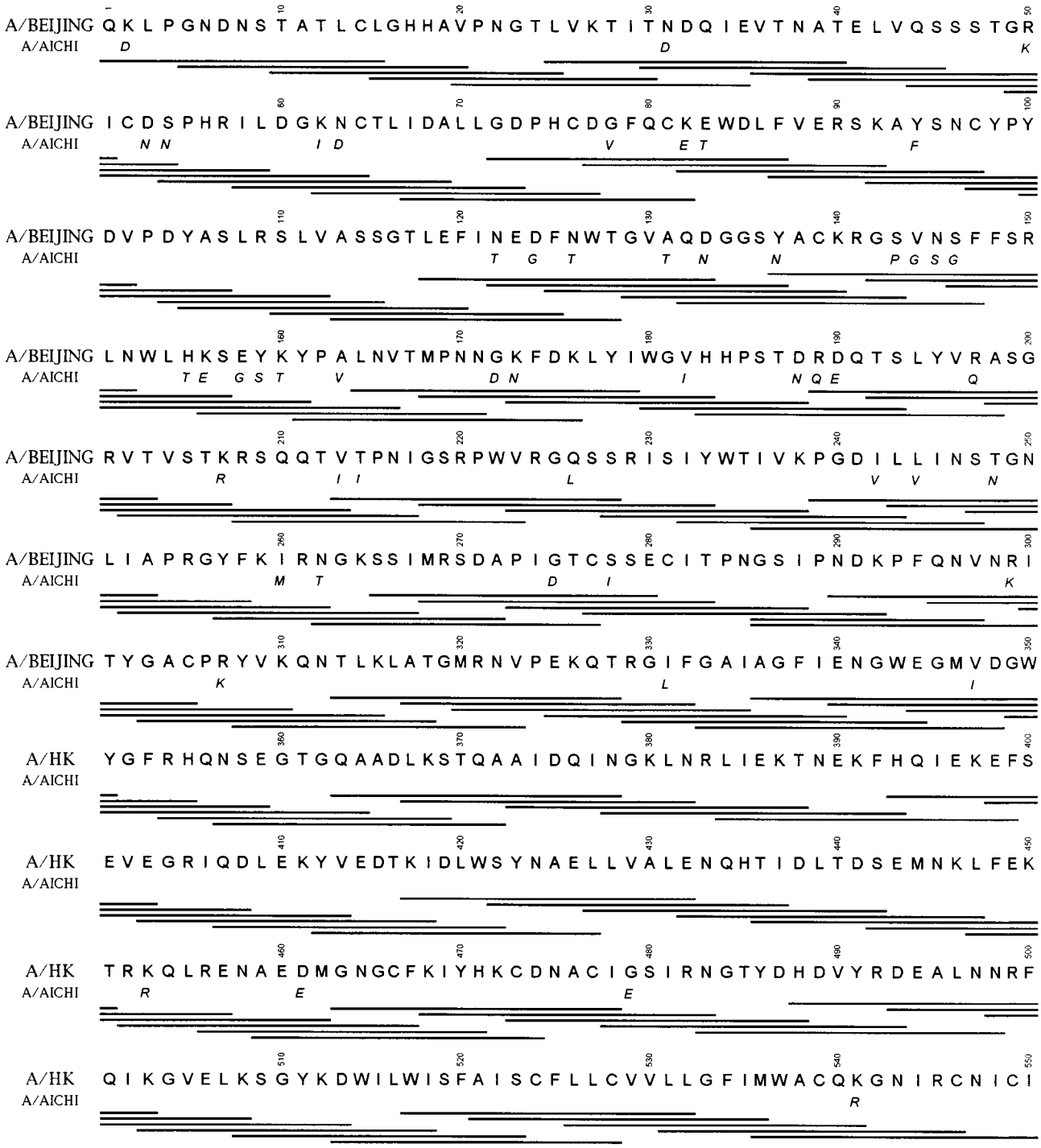


FIG. 1. Sequences of HA from A/Beijing/32/92 and peptides used in this study. The amino acid sequence used for peptide synthesis, based on the putative sequence of HA0, is shown. The HA1 subunit (residues 1 to 328) is based on A/Beijing/32/92. The HA2 subunit (residues 330 to 550) is based on the closely related A/Hong Kong/90, as no sequence information on the HA2 subunit of A/Beijing/32/92 was available at the time of synthesis. Sequence differences between A/Beijing/32/92 and A/Aichi/68 HAs (in italics below main sequence) are indicated. A/Beijing, A/Beijing/32/92 HA; A/Aichi, A/Aichi/68 HA; A/HK, A/HongKong/90 HA.

supernatants and purified with protein A-Sepharose. Autologous irradiated (3,000 rads) PBMC ($4 \times 10^4$/well) were incubated for 30 min with 10 μg of antibody per ml before the addition of antigen (1 and 5 μg of individual peptides per ml). After 1 h of incubation at 37°C in round-bottomed 96-well plates in complete medium, T cells ($4 \times 10^4$/well) were added. The cells were cultured, pulsed, and harvested as described above.

**Cross-reactivity of T-cell lines among a panel of influenza A viruses.** The following freeze-dried viruses were kind gifts of P. Chakraverty, Public Health Laboratory Service, Colindale, London, United Kingdom: H1N1, A/Formosa/1/47, A/Taiwan/1/86, and A/Fiji/2/88; H2N2, A/Singapore/1/57; and H3N2, A/Victoria/3/75, A/Bangkok/1/79, and A/England/427/88. Between 1 and 10 hemagglutinating units of virus was preincubated with autologous irradiated APCs for 1 h, which were then cultured with T cells as described above.

**Fluorescence flow cytometry.** T-cell lines were stained directly with saturating concentrations of fluorescein isothiocyanate-conjugated murine monoclonal antibodies anti-Leu-4 (CD3), anti-Leu-3a (CD4), and anti-Leu-2a (CD8) (Becton

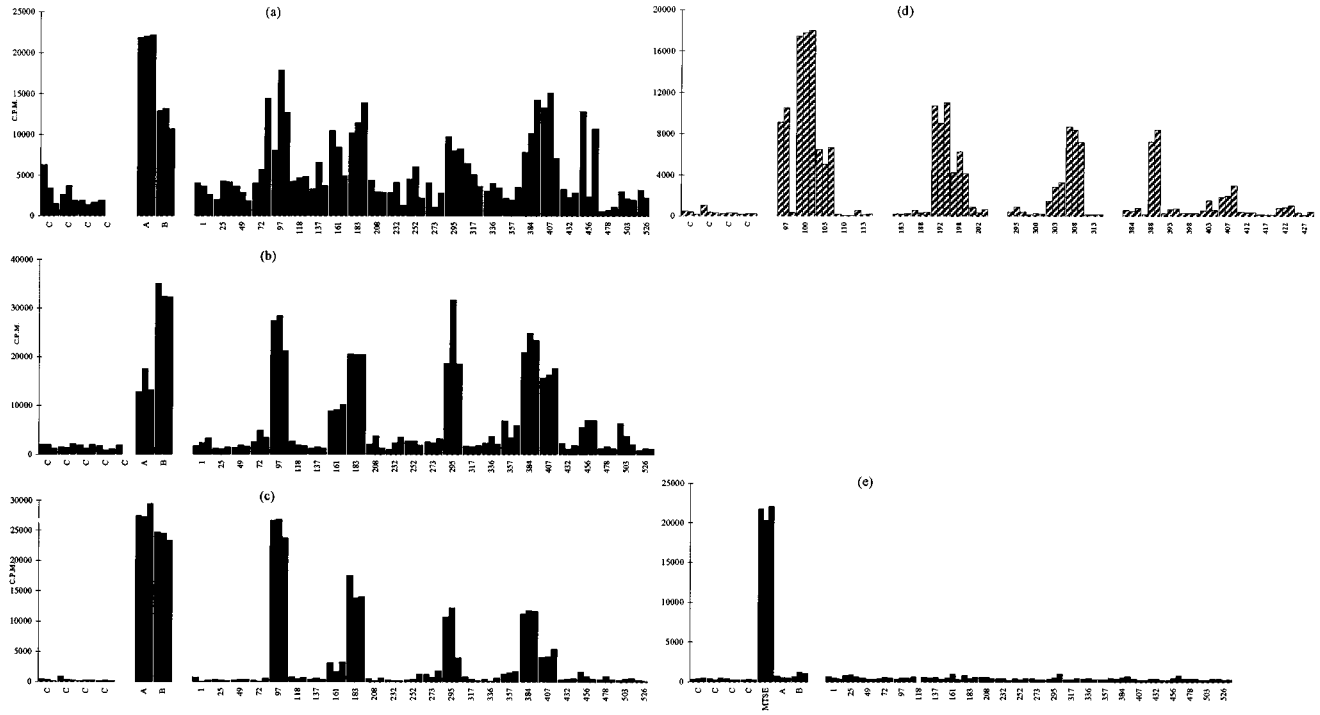


FIG. 2. Development of HA response by $CD4^+$ T-cell line derived from donor A and selected for 3 weeks with A/Beijing/32/92 HA (a to d), plus response of control MTSE-specific $CD4^+$ T-cell lines to HA and peptides (e). T-cell proliferation in response to A/Aichi/68 HA, A/Beijing/32/92 HA and peptide pools was tested at 7 (a), 14 (b), and 21 (c) days by using irradiated autologous prepulsed PBMC as APCs. At 48 h, the T-cell lines were pulsed with [$^3$H]thymidine, and they were harvested 16 h later. The *y* axis represents individual data for each well of a triplicate; C, control (proliferative response of T-cell line to complete medium and autologous APCs); A, response of T-cell line to A/Aichi/68 HA (1.0 μg/ml); B, response to A/Beijing/32/92 HA (0.1 μg/ml). Peptide pools are labelled according to the number of the first residue of the first peptide in the pool (five peptides per pool, 10 μg of each peptide per ml). The *y* axis represents counts per minute. (d) More-detailed mapping with single peptides (10 μg/μl) conducted at day 28. (e) Response of MTSE-specific T-cell line to HA and peptides used in this study. The $CD4^+$ T-cell line was derived from a donor nonresponsive to A/Beijing/32/92 HA by stimulating PBMC with 5 μg of MTSE per ml. Bar MTSE, response to 5 μg of MTSE per ml.

Dickinson Immunocytometry Systems, San Jose, Calif.). Viable cells, identified by their ability to exclude propidium iodide, were analyzed by flow cytometry with an Epics Profile II (Coulter Electronics, Luton, Beds, United Kingdom). The cell population was analyzed by gating on the volume and light scatter characteristics.

## RESULTS

**Subjects.** The panel of donors with a history of influenza during November and December 1993 (recent influenza virus donors) had a wide range of MHC class II haplotypes (Table 1). One pair, donors A and B, shared MHC HLA DRB1* and DQB1* haplotypes (DRB1*0101,11, DRB3*01/02/03, and DQB1* 0301,05). A second pair, donors C and D, had identical low-resolution MHC class II types (DRB1*04,15, DRB4*01, DRB5*01, and DQB1*0301,0601/2) and differed only in HLA DRB1*04 subtype. Three donors (D, G, and I) did not proliferate in response to HA from A/Aichi/68 during the summer of 1993 but recognized both A/Beijing/32/92 and A/Aichi/68 HAs after influenza-like illness in November and December 1993 (not illustrated).

The second panel of donors, with no history of influenza during the preceding 4 years (control donors), also had a range of MHC class II haplotypes, although four shared the DRB1* 0101 allele (Table 1). Donor O shared low-resolution HLA DR and DQ types with donor E, who had a history of recent influenza.

In total, of the 18 donors in the two groups, 7 shared the DRB1*0101 DQB1*05 haplotype, 5 shared the low-resolution HLA DRB1*04 type (but had several different subtypes), 4 shared the DRB1*15 haplotype, 3 shared the DRB1*13 haplotype, 2 shared the DRB1*07 haplotype, and 2 shared the DRB1*11 haplotype. In addition, the responses of donors with HLA DRB1*0102, -0103, -03, -08, -10, and -12 haplotypes were examined.

**Lack of mitogenicity of HAs and peptides.** The antigens used in this study were tested for mitogenicity by using a human $CD4^+$ T-cell line specific for *Mycobacterium tuberculosis* soluble extract (MTSE). No mitogenicity (greater than three times the geometric mean) was induced by A/Beijing/32/92 or A/Aichi/68 HA or any of the synthetic peptides (Fig. 2e).

**Characteristics of T-cell lines.** T-cell lines derived from the recent influenza virus donors and selected with A/Beijing/32/92 HA expanded at least fourfold in culture over 3 weeks, in contrast to those derived from the control donors, which, with the exception of donor Q's T-cell line, showed no significant expansion (data in Table 1).

Analysis of two T-cell lines by flow cytometry showed that the ratio of $CD4^+$ cells to $CD8^+$ cells progressively increased from days 7 to 21. Six T-cell lines tested at 21 days were >90% $CD3^+$, 78 to 88% $CD4^+$, and 2 to 10% $CD8^+$ (not illustrated).

Examples of the proliferative responses obtained from donor A at 7, 14, and 21 days in culture are shown in Fig. 2 (a to c). The dominant responses are to peptide pools 97-128, 183-217, 295-328, 384-418, and 407-442, while the background proliferation progressively drops with time. In general, the patterns of response to major epitopes remained stable, though there was some loss of response to minor epitopes. Figure 2d shows the results of more-detailed analysis of epitope recognition by this line using single peptides at 28 days. Because of the increased ratio of $CD4^+$ T cells and low background pro-

liferation, we chose to analyze peptide specificity at 21 days whenever possible.

**Responses of CD4+ T-cell lines derived from unrelated donors with a history of recent influenza to HA and peptides.** The recent influenza virus donors responded to both A/Beijing/32/92 and A/Aichi/68 HAs (Fig. 3). Marked differences in patterns of peptide recognition were seen between individual T-cell lines from donors differing in MHC class II haplotype. Without exception, every CD4+ T-cell line mounted a significant proliferative response to the HA2 subunit.

When results for individual recent influenza virus donors during the second and third weeks of culture were combined, we found that almost the entire protein sequence as represented by the peptide pools could induce proliferative responses. Three regions of HA, represented by peptide pools 97-128, 295-328, and 407-442, were recognized by 10 of 12, 12 of 12, and 11 of 12 donors, respectively, during the second and/or third weeks. A further three regions (pools 183-217, 357-393, and 384-418) were recognized by 8 of 12 donors. Studies with single peptides within the dominant regions showed that the responses usually localized to identical individual peptides or a pair of overlapping peptides, representing regions of HA that have been structurally conserved over many years among H3 influenza A viruses (not illustrated). Pool 407-442 was found to contain at least two immunogenic regions.

**Responses of CD4+ T-cell lines from adult donors with no history of influenza during the preceding 4 years to HA and peptides.** The responses of the six control donors (M to R) were, in general, to regions of HA similar to those of the recent influenza virus donors, though not as strong (Fig. 4). Once again, every T-cell line responded to the HA2 subunit. Pools 97-128, 295-328, and 407-442 were recognized by four, five, and six of six donors, respectively.

**Influence of MHC class II on T-cell recognition of HA peptides.** It is striking that the responses of donors A and B (sharing DRB1*0101,11, DRB3*01/02/03, and DQB1*0501,0301) to the peptide pools were very similar; both responded to pools 97-128, 183-217, 295-328, and 407-442, and studies with single peptides demonstrated that the responses were localized to identical peptides or pairs of overlapping peptides within the pools (not illustrated). Donors C and D (DRB1*04,15, DRB4*01, DRB5*01, DQB1*0301,0601/2) differed in DRB1*04 subtype (0401 and 0408, respectively), but both T-cell lines responded strongly to pools 1-25, 25-59, 97-128, 273-305, 295-328, 407-442, and 456-488. An additional response to 252-283 was made by donor D, and again, when single-peptide studies were undertaken, dominant responses were found to be directed to identical individual peptides. Donors E and O, who differed in history of exposure to influenza virus but shared low-resolution HLA DRB1*01,04, DRB4*01, and DQB1*03,05 types, made strong responses to identical peptide pools, though there were differences in the relative strengths of their responses.

Similarities in patterns of response were also seen between individuals who shared a single MHC class II haplotype. This was particularly evident for HLA DRB1*0101 for subject E, who shared DRB1*0101 and DQB1*0301,05 with subjects A and B and responded to pools 97-128, 183-217, 295-328, 384-418, and 407-442. The responses of cell lines from subjects M, O, and R, who had no history of recent influenza and who shared the MHC class II alleles DRB1*0101 and DQB1*05, were very similar to those of subjects A, B, and E, with all six lines responding strongly to peptide pools 97-128, 295-328, and 407-442. Subject Q (DRB1*0101,12, DRB3*01/02/03, DQB1*0301,05) responded to pools 295-328 and 407-442 but not to pool 97-128.

In contrast, marked differences were seen in response patterns of donors with different subtypes of HLA DRB1*01: neither donor I (DRB1*0102) nor donor N (DRB1*0103) followed the pattern seen for the DRB1*0101 donors.

**Results of studies with anti-MHC class II antibodies.** The results of the MHC restriction studies using antibodies to MHC class II on two T-cell lines are shown in Fig. 5. The majority of responses by both donors were inhibited by anti-HLA-DR antibody and were therefore HLA DRB1* restricted. Interestingly, donor D showed some increase in proliferative response to peptide pairs 20/25, 97/100, and 303/308 following preincubation with anti-HLA-DP antibodies.

**Influenza virus cross-reactivity.** Six T-cell lines from the recent influenza virus donors, including all three donors (D, G, and I) who were nonresponsive to A/Aichi/68 HA prior to November or December 1993, were examined for cross-reactivity among a panel of H1, H2, and H3 influenza A viruses (Fig. 6). All were cross-reactive, although the relative strengths of responses to H1, H2, and H3 viruses varied considerably. T-cell line I showed a stronger response to H3 viruses than to the H1 or H2 viruses. This T-cell line was dominated by responses to HA2. Studies with single peptides localized the response to regions 384 to 399, 403 to 422, 427 to 442, and 463 to 483 (not illustrated). In three of these four regions, radical changes (residue 388, T to M; 404, G to K and R; 412, Y to K; 417, F to K; 430, A to L; and 440, T to H) which probably account for these observed differences in response occur between H3 and H1 and H2 influenza A viruses.

## DISCUSSION

We report the first major study of the human CD4+ T-cell repertoire of responses to HA after recent natural infection. CD4+ T-cell lines were derived from a panel of 12 unrelated MHC class I- and II-haplotyped adult donors with a history of influenza during November and December 1993 (recent influenza virus donors) and a panel of 6 adult donors who responded to HA but who had no history of influenza in the past 4 years (control donors). CD4+ T-cell lines were selected by using full-length HA from A/Beijing/32/92 and epitope recognition mapped by using a panel of 16-mer peptides, overlapping by 11 residues, covering the entire sequence of HA1 and HA2. Cross-reactivity was investigated by using the original 1968 H3 influenza A virus HA and a panel of influenza A viruses. Results have been interpreted in the context of the donors' MHC class II haplotypes.

HA was highly immunogenic in all cases. We expected MHC class II to play an important role in HA epitope selection in association with an individual's exposure history and other factors. It is remarkable, however, that proliferative responses by the short-term T-cell lines from unrelated donors A and B, who are both DRB1*0101,11, DRB3*01/02/03, DQB1*0301,05, were both directed predominantly to peptide pools 97, 183, 295, 384, and 407, with only some quantitative differences. Analysis using single peptides revealed that their responses were to identical peptides or pairs of overlapping peptides (not illustrated). Donors E and O, who share low-resolution HLA DR and DQ types but differ in exposure history, also recognized identical peptide pools. This similarity in pattern of response was not limited to donors with such close MHC class II matches, as all seven donors who expressed DRB1*0101 and DQB1*05 alleles, irrespective of differences in their history of exposure to influenza virus and differences in other HLA class

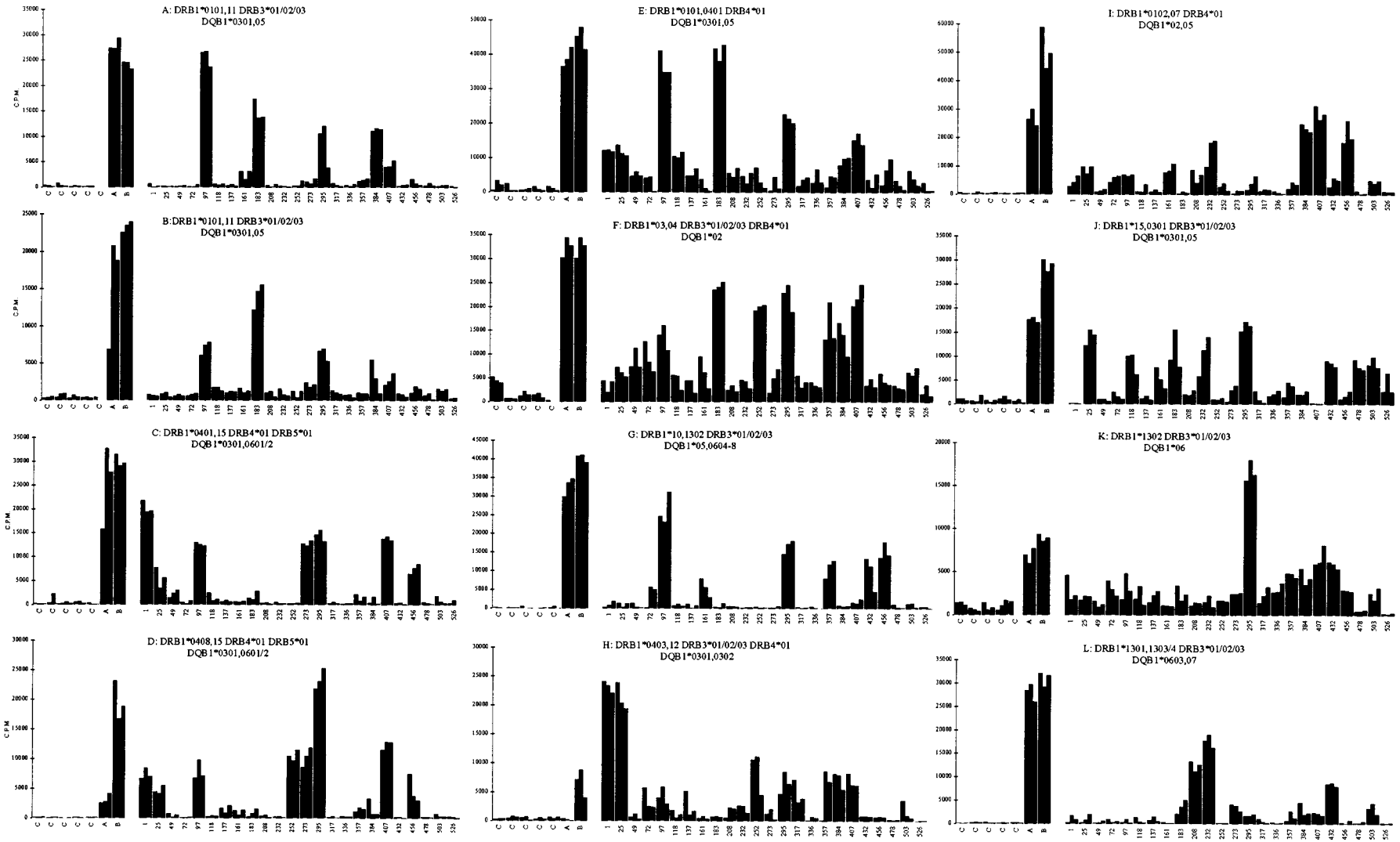


FIG. 3. HA response by CD4$^+$ T-cell lines from 12 unrelated donors (A to L) with a history of recent influenza and selected for 2 or 3 weeks with A/Beijing/32/92 HA. A/Beijing/32/92 HA, A/Aichi/68 HA, and peptide pools were presented to T-cell lines as described for Fig. 2. The class II MHC types of the donors are shown.

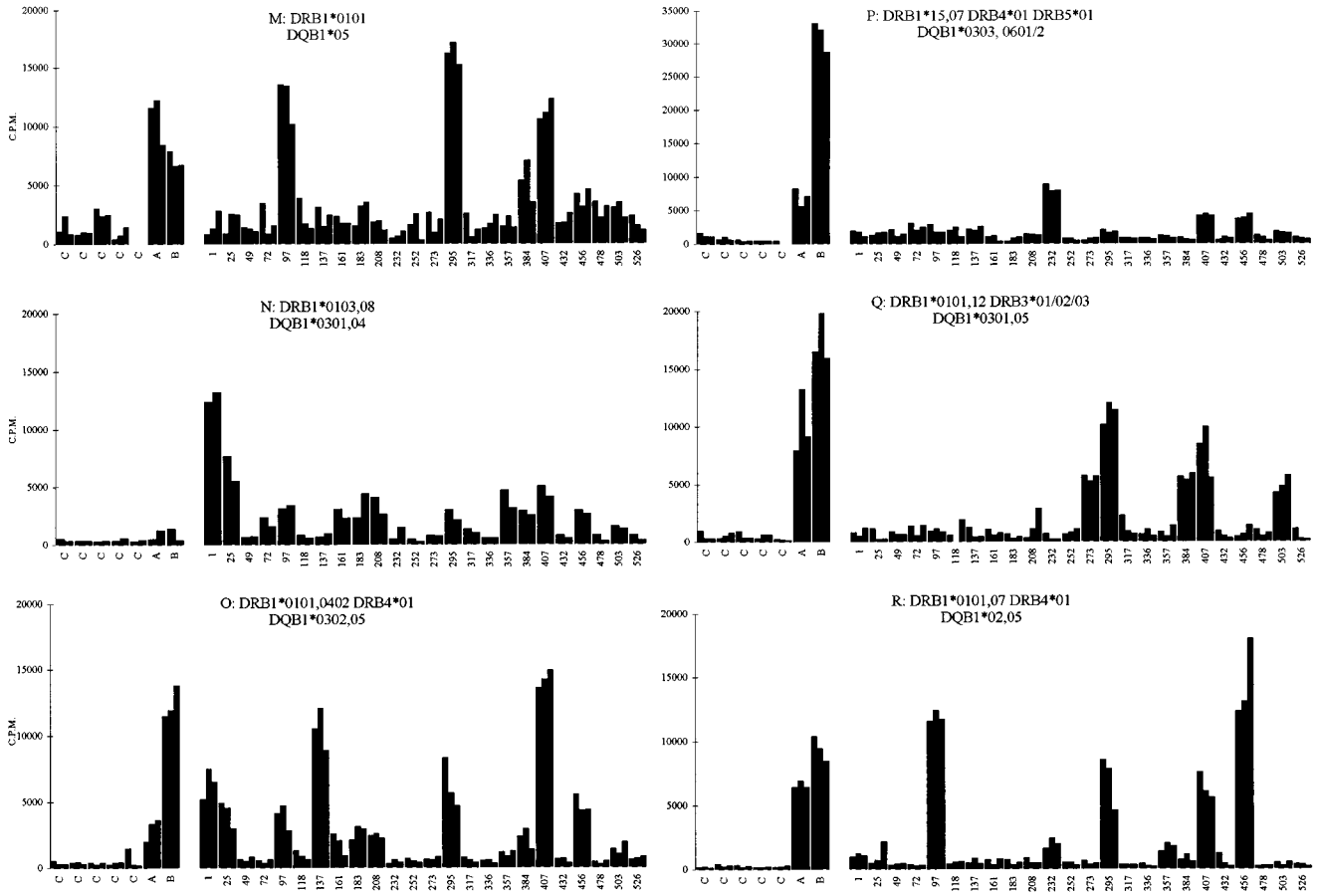


FIG. 4. HA responses of CD4+ T-cell lines from donors with no history of influenza during the last 4 years. For details, see the legend to Fig. 2. Three donors had previously been frequently exposed to influenza virus, donors N and P, who were practicing physicians, and donor O, who had worked extensively with H3 influenza A viruses 10 years previously.

II alleles, showed similar patterns of T-cell recognition (except for donor Q, who failed to respond to pool 97-128).

As predicted by peptide binding studies (11, 12), the subjects who expressed other subtypes of HLA DR1, donors I (DRB1*0102) and N (DRB1*0103), in peptide recognition differed from both the DRB1*0101 donors and each other. There are only limited structural differences between these alleles (changes of V to A at residue 85 and of G to V at residue 86 for DRB1*0102 and changes of L to I at residue 67, Q to D at residue 70, and R to E at residue 71 for DRB1*0103). Six donors shared the low-resolution HLA DRB1*04 haplotype; two of them, donors C (DRB1*0401) and D (DRB1*0408), also shared DRB1*15 and had very similar patterns of peptide responsiveness. The variations between these donors may reflect the slight difference between DRB1*0401 and DRB1*0408 (change at position 71 from K to R). Identifiable patterns of response are also evident for HLA DRB1*07, with donors P, I, and R all responding strongly to pool 456-488.

Most interestingly, HA2 (residues 330 to 550), which has been regarded as largely nonimmunogenic (5), induced strong responses in every donor. Furthermore, with the exception of the N and C termini of HA2, every peptide pool representing HA2 induced a response in at least 3 of 12 recent influenza virus donors. One conserved region (residues 407 to 442) induced responses in 11 of 12 recent influenza virus donors during the second or third week in culture; this region is unchanged among H3 influenza A viruses and has homology with H1 and H2 viruses. Two additional highly conserved regions (residues 357 to 393 and 384 to 418) were recognized by 8 of 12 recent influenza virus donors. We are aware of only one preliminary study of the human CD4+ T-cell response to HA2: unselected PBMC were derived from 14 donors, who were not tissue typed, and limiting dilution analysis showed T-cell responses to at least three regions of HA2 (29). One murine study has demonstrated cross-reactive CD4+ T-cell recognition of HA2 after nasal infection with live virus (though no epitopes were determined) (18). A second murine study demonstrated responses to HA2 following vaccination with HA2 alone and characterized two epitopes (residues 425 to 437 and 499 to 511) (17).

Regarding the HA1 subunit (residues 1 to 328), two regions, residues 97 to 128 and 295 to 328, were the most frequently recognized, inducing responses in 10 of 12 and 12 of 12 recent influenza virus donors, respectively. Studies with single peptides localized the majority of responses to residues 100 to 115 and 306 to 323, which are conserved among H3 influenza A viruses (not illustrated). The second region has sequence homology with H1 and H2 viruses and is closely related to the dominant epitope originally described by Lamb et al. (who used a different panel of peptides) (19, 21). One peptide pool which contained a variable region within the H3 influenza A viruses, pool 183-217, induced responses in 8 of 12 donors. The majority of responses localized within this region were to residues 192 to 212, which have been relatively conserved during

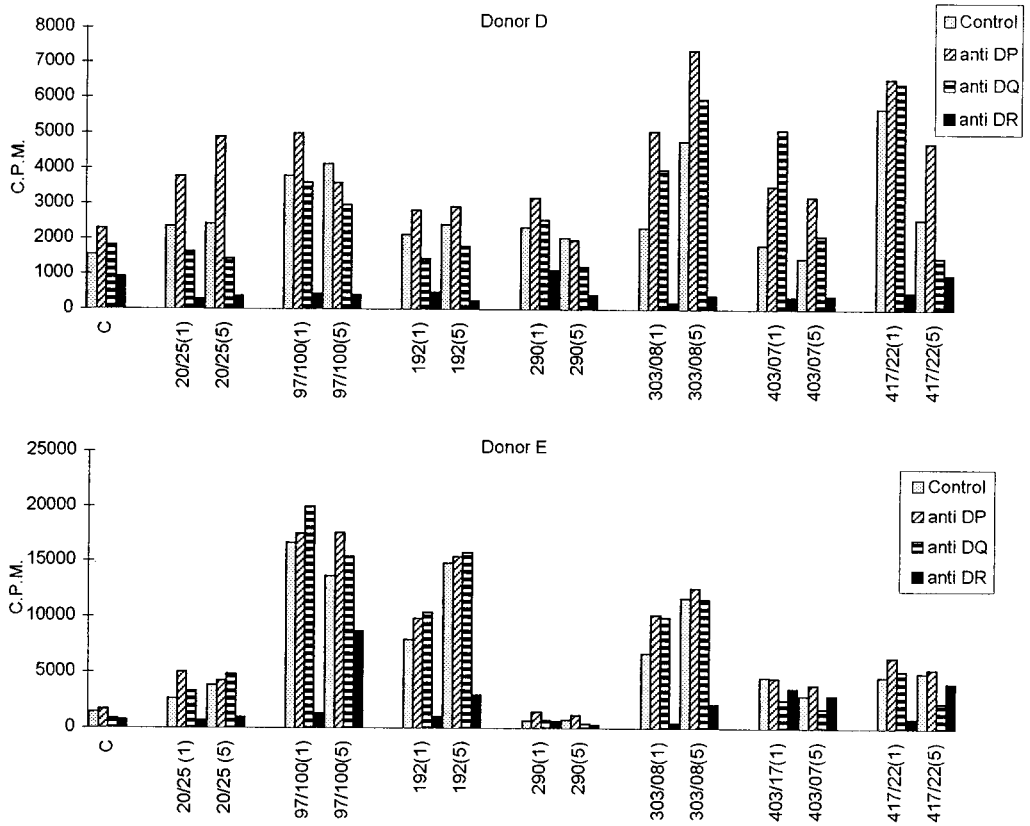


FIG. 5. Effect of MHC class II blocking antibodies on the response to dominant peptides. Autologous PBMC were incubated with 10 μg of anti-HLA-DP, anti-HLA-DQ, or anti-HLA-DR antibodies per ml or with medium (control). Individual peptides or pairs of peptides (1 and 5 μg/ml) were added to the PBMC and incubated at 37°C for 1 h prior to addition of responder $CD4^+$ T cells. Further experimental details are described for Fig. 2. Numbers on the *x* axis refer to the individual peptides or pairs of peptides; numbers in parentheses refer to the dose of peptide (in micrograms per milliliter).

the past decade (changes: residue 193, S to N; 197, Q to R; 201, R to K; and 207, K to R). Responses of $CD4^+$ T-cell lines derived from control donors M to R were examined as a comparison of results after 3 to 6 months and longer-term $CD4^+$ T-cell memory. The two groups recognized similar peptide pools, though the responses of the control panel were generally weaker. Interestingly, the control lines demonstrated an expansion in vitro which was severalfold lower than those of the recent influenza virus donors (Table 1). The most likely explanation is a lower T-cell precursor frequency, implying a decline in circulating $CD4^+$ T-cell memory after infection. A similar decline in circulating influenza virus-specific $CD8^+$ T-cell memory has been reported (23), and both findings are in agreement with early epidemiological surveys which showed protection from influenza virus following natural infection declining after 4 years (28).

It is interesting that viral infection induces recognition of several conserved HA epitopes by a large proportion of the population. Our findings concur with the original human $CD4^+$ T-cell studies (which were limited to single donors) in which, with the exception of one clone specific for a variable region of an H2 influenza A virus (8), the responses were either cross-reactive between H3 influenza A viruses (and sometimes H1 and H2 viruses) (14, 20) or localized to conserved regions of the HA1 subunit (21). This contrasts with recent murine studies of live-virus infection which have demonstrated strong $CD4^+$ T-cell responses to epitopes closely related to the antibody-neutralizing sites (3, 4, 10, 15, 24, 32).

Several explanations for these apparent species differences are possible. First, our donors may have been infected with viral strains differing from A/Beijing/32/92. However, the majority of influenza A virus strains isolated in London in November and December 1993 were similar to A/Beijing/32/92: four conservative substitutions (residue 75, H to N; 145, N to K; 201, R to K; and 208, R to K), three intermediate substitutions (residue 189, R to S; 214, T to I; and 276, T to N), and three nonconservative substitutions (residue 157, S to L; 219, S to F; and 226, Q to L) were recorded (12a). These changes offer an explanation for the low frequency of response to pools 137-171 and 208-243 which was seen but do not account for the dominance of $CD4^+$ T cells recognizing HA regions conserved within the H3 subtype. Alternatively, repeated exposure to influenza A viruses in humans may bias the $CD4^+$ T-cell response towards recognition of conserved HA epitopes. There is evidence for this phenomenon in the human B-cell response to HA (27).

We were fortunate that three donors (D, G, and I) failed to mount detectable proliferative responses to A/Aichi/68 HA during the summer of 1993. Following influenza-like illness in November and December 1993, all three donors made strong responses to both A/Aichi/68 and A/Beijing/32/92 HAs, demonstrating the induction of significant cross-reactivity.

Finally, differences in antigen presentation between I-A, which is equivalent to human HLA DQ, and HLA DR (equivalent to murine I-E) may account for these findings. Interest-

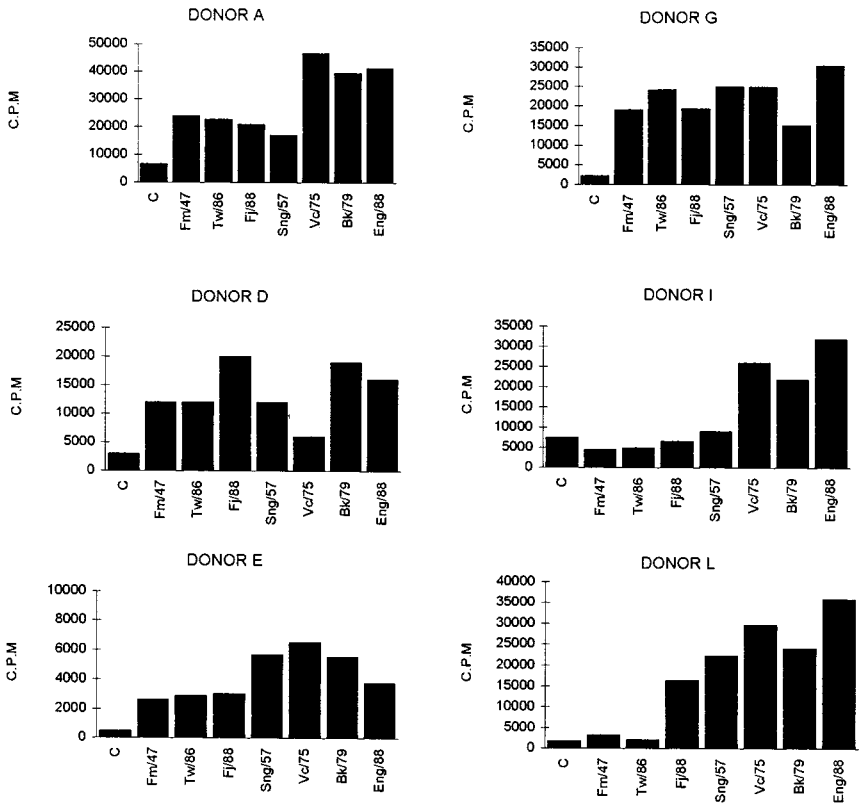


FIG. 6. Responses of six $CD4^+$ T-cell lines to a panel of influenza A viruses. Autologous irradiated PBMC were prepulsed for 1 h with 1 to 10 hemagglutinating units of virus prior to the addition of 3-week $CD4^+$ T-cell lines selected by A/Beijing/32/92 HA. C, control (T cells plus APCs). Viruses: Fm/47, A/Formosa/1/47; Tw/86, A/Taiwan/1/86; Fj/88, A/Fiji/2/88 (all H1N1); Sng/57, A/Singapore/1/57 (H2N2); Vc/75, A/Victoria/3/75; Bk/79, A/Bangkok/1/79; and Eng/88, A/England/427/88 (all H3N2).

ingly, Burt and coworkers have reported I-E-restricted responses directed towards more-conserved regions of HA1 (10).

Influenza virus vaccination achieves a reduction of approximately 50% in hospital admissions due to influenza and pneumonia among the elderly during an epidemic (25). Because of the persistence of antigenic variation in HA, complete protection is strain specific and generally short-lived. The partial protection obtained from vaccination may be due in part to the induction of a cross-reactive $CD4^+$ T-cell response. This is supported by our preliminary observations of the human $CD4^+$ T-cell response to HA following vaccination. As a few conserved regions of HA were recognized by a large proportion of our donors, it may be possible to design specific $CD4^+$ T-cell vaccines to boost cross-reactive protection.

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