DIVERSITY OF THE CLASS II (I-A^k/I-E^k)-RESTRICTED T CELL REPERTOIRE FOR INFLUENZA HEMAGGLUTININ AND ANTIGENIC DRIFT

Six Nonoverlapping Epitopes on the HA1 Subunit Are Defined by Synthetic Peptides

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Estimates of diversity in the class II-restricted T cell repertoire for protein antigens are derived from studies of the recognition specificity of CD4$^+$ T cell clones for model protein antigens:myoglobin (1, 2), cytochrome *c* (3), or lysozyme (4, 5), and in each instance it has been found that T cells focus on limited regions of the molecule in a haplotype-specific manner. Similar constraints on the immune repertoire are evident from studies with phylogenetically distant proteins such as staphylococcal nuclease (6), thereby arguing against the considerations of self-tolerance to conserved regions of a molecule. Such limited diversity is consistent with the current paradigm of antigen processing and presentation; "processed antigen" or peptides bind selectively to class II molecules of the APC (7-9) and determine the expressed T cell repertoire. The findings that antigenic peptides of lysosyme (10) or ovalbumin (11) bind to purified class II molecules in vitro with specificities that correlate with their known MHC restriction provide convincing evidence for the determinant selection model. Moreover, the ability of antigenic peptides from unrelated proteins to compete for antigen presentation in association with the same MHC restriction element (12) is consistent with their binding to a single receptor site.

In a recent study of the antigenic specificity of CD4$^+$ T cell clones from the H-2^k haplotype for influenza hemagglutinin (HA)[1] (13), we reported a complex recognition pattern for natural variant viruses from which we concluded that (*a*) CD4$^+$ T cells are sensitive to the amino acid substitutions that have featured in HA during antigenic drift in response to antibody selection, and (*b*) extensive diversity exists in the T cell memory repertoire. A total of 12 distinct antigenic specificities were evident from the ability of CD4$^+$ clones to discriminate between natural variant viruses, and a comparison of the primary amino acid sequences of mutant HAs identified a major T cell specificity, centered on residue HA1 54 (13), that was confirmed by the recognition of the synthetic peptides HA1 48-68 (13) and HA1 53-63 (14). The recognition specificities of the remaining CD4$^+$ clones could not be predicted

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from their reactivites with H3N2 subtype viruses, arguing that rather than exhibiting distinct specificities, these clones may recognize closely overlapping HA1 sequences. Hence the differential patterns of recognition could reflect alterations in the processing and expression of a common epitope in the mutant HAs.

Here we provide evidence for extensive diversity in the class II-restricted repertoire for influenza HA: I-A^k-restricted T cell clones are shown to recognize four distinct and nonoverlapping regions of HA, HA1 53-63, HA1 68-83, HA1 120-139, and HA1 269-288; each region has featured in antigenic drift and a structural correlation can be made between the primary sequence differences of natural variants and the recognition specificities of individual $CD4^+$ clones. In contrast, two I-E^k-restricted clones were crossreactive, failed to discriminate between H3 subtype viruses, and recognized conserved regions of HA1 sequences 226-245 and HA1 246-265.

Materials and Methods

Synthetic Peptides. Peptides were synthesized according to the sequence of HA1 from X31 influenza virus (Fig. 1) by a manual solid-phase procedure (15) modified to allow simultaneous multiple synthesis (16) using fluoroenylmethoxy-carbonyl (Fmoc) amino acids (17). Briefly, Pepsyn resin (0.3 mequiv/g) (Milligen, Cambridge, UK) was derivatized with Fmoc norleucine internal reference standard and pentafluorophenyl-4-hydroxymethyl phenoxyacetate linker agent (Milligen). COOH-terminal aminoacids were coupled to resin as symmetrical anhydrides using Fmoc aminoacids (eightfold molar excess) (Milligen) and dicyclohexylcarbodiimide (Sigma Chemical Co., Poole, UK). 150-mg aliquots of resin were sealed into polypropylene mesh bags (40-mm^2) ("Propyltec" PP74; P and S Filtration Ltd., Lancs, UK). Peptides were assembled using Fmoc amino acid pentafluorophenyl esters (fourfold molar excess) (serine and threonine were used as esters of 3,4 dihydro-3-hydroxy-4-oxo-1,2,3 benzotriazine), together with equimolar quantities of 1-hydroxybenzotriazole catalyst and 3,4 dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine, which enabled qualitative measurement of the progress of the reaction (18). Fmoc deprotection was achieved using 20% piperidine. Cleavage of peptides from resin and removal of *t*-butyl-(Ser, Thr and Tyr), *t*-butylester-(Asp and Glu), trytyl-(Cys), 4-methoxy-2,3,6, trimethylbenzene sulphonyl-(Arg) and *t*-butyloxycarbonyl-(Lys) side-chain protecting groups were performed simultaneously in 95% trifluoroacetic acid (TFA) and 5% water or mixture of appropriate scavengers. After filtration and rotary evaporation, the residue was washed in methanol (2×) and diethylether (5×) to remove residual TFA and scavengers. Peptides were dried and stored dessicated at 4°C. Correct composition of each peptide was confirmed by amino acid analysis after hydrolysis in 6 N HCl at 110°C for 24 h. No further purification of peptides was performed before use in T cell assays.

Viruses. All viruses were grown in the allantoic fluid of embryonated hen eggs and viral titers were determined by HA assays, expressed as HAU/ml. Viruses were stored at −70°C until use. X31 is a recombinant between A/Aichi/2/68 and A/PR/8/36 expressing Hong Kong glycoproteins (H3N2) and PR8 internal proteins (19). Natural variant viruses were all of the H3N2 subtype and were isolated from major influenza outbreaks between 1968 and 1984. A/Duck/Ukraine/63 F is an avian variant virus (H3N8). Laboratory mutants M1 and 184 were produced by growing parental X31 virus in eggs in the presence of mAb. The amino acid sequences of HA1 from variant viruses were deduced from the nucleotide sequences of their RNA genes (our unpublished results) or taken from published data (20, 21).

Mice. CBA/Ca, B10.(AQR) and BALB/c strains of mice were bred under specific pathogen-free conditions at NIMR. B10A(4R) mice were obtained from Harlan Olac Ltd., Bicester, UK. Mice were used when 3-4 mo old.

T Cell Lines and Clones. The generation of the HA-specific T cell clones used in this study has been described previously (13). Briefly, clones were established from lines from the spleen cells of individual CBA/Ca (H-2^k) mice primed by intranasal infection with X31 virus. T cell clones were maintained by restimulation with X31 virus (100 HAU/ml) and x-irradiated

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syngeneic spleen cells (2×10^6/ml) every 10–12 d followed by 5% IL-2-containing supernatant (from Con-A-activated rat spleen cells) and feeders 3–4 d later.

T Cell Proliferation Assays. The peptide specificities of the T cell clones were determined by T cell proliferation assays, performed in triplicate in 96-well tissue culture plates 10–12 d after the previous antigen stimulation. T cells (2×10^4) were incubated with x-irradiated syngeneic spleen cells (4×10^5) together with virus, peptide, or medium alone in 10% FCS at 37°C for 72 h. Proliferation was assessed by determining the incorporation of [^{3}H]thymidine (1 μCi/ml) added during the last 6 h of assay.

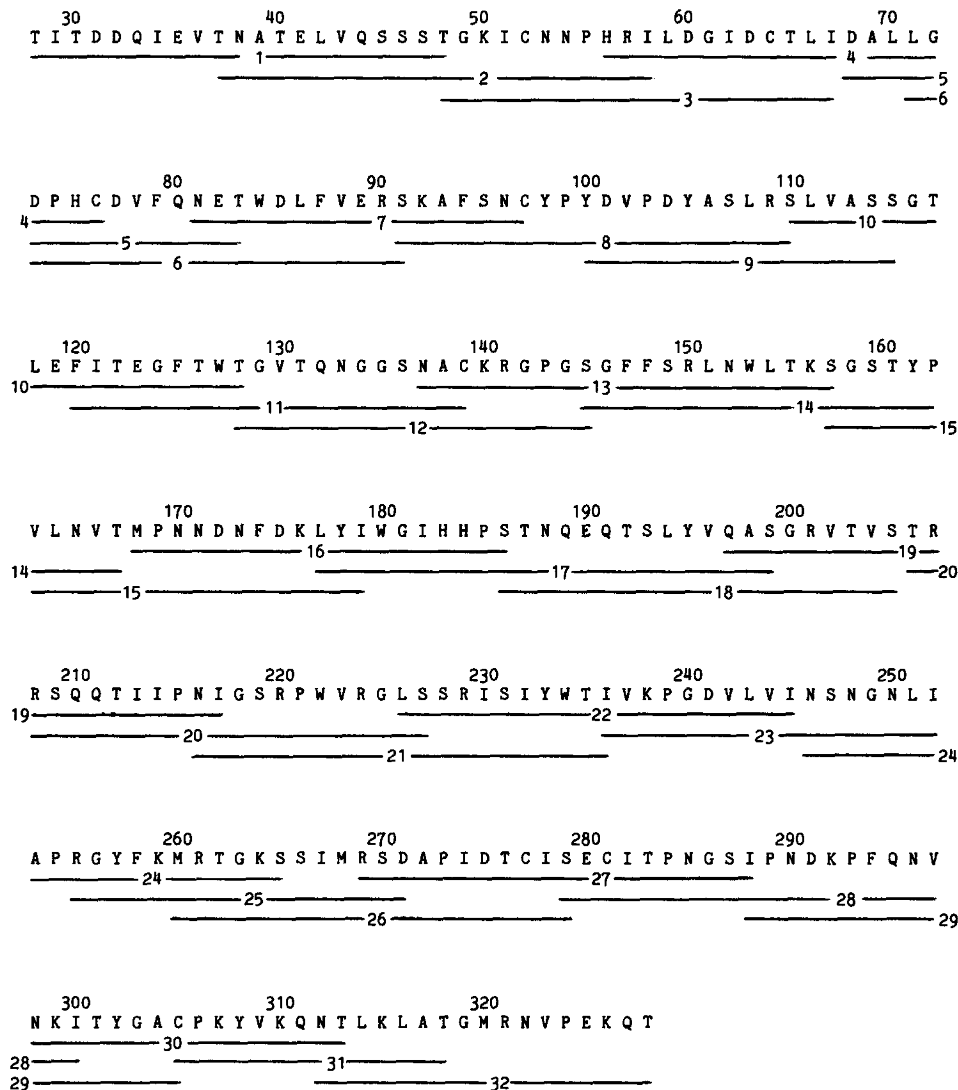

FIGURE 1. Amino acid sequence of the HA1 subunit of influenza HA (HA1 28–328) based on the H3N2 recombinant virus X31. Synthesized peptides and their code numbers are indicated.

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Results

Recognition of Synthetic Peptides of HA1 28–328. A large number of $CD4^+$ T cell clones derived from CBA mice infected with X31 (H3N2) influenza virus responded in T cell proliferation assays to X31 virus, to BHA, which includes the complete HA1 polypeptide sequence, and also to a tryptic fragment of HA, HA1 28–328 (13). The specificity of one group of clones was further mapped to HA1 53–63 (13, 14). The HA-specific T cell clones characterized in this present study are representatives of five additional distinct antigenic groupings based on their ability to discriminate between a panel of H3N2 natural variant viruses (reference 13 and Tables II–VI). In order to determine the sequences within HA1 recognised by these clones, each was screened for its proliferative response against 32 overlapping synthetic peptides (Fig. 1) covering the complete aminoacid sequence of HA1 28-328 of X31 virus.

Fig. 2 shows the responses of four of these T cell clones against each individual peptide, tested in a single assay at an identical concentration. Clones 1.3, 4.4.4, 3F10, and 1.99 responded preferentially to single, unique HA1 peptides representing amino acid sequences 68–83, 120–139, 226–245, and 246–265, respectively (Figs. 2, *a–d*). Clone 1.4 was only stimulated by peptide HA1 269–288 (data not shown). The specificities of the T cell clones were confirmed in dose-response experiments, shown

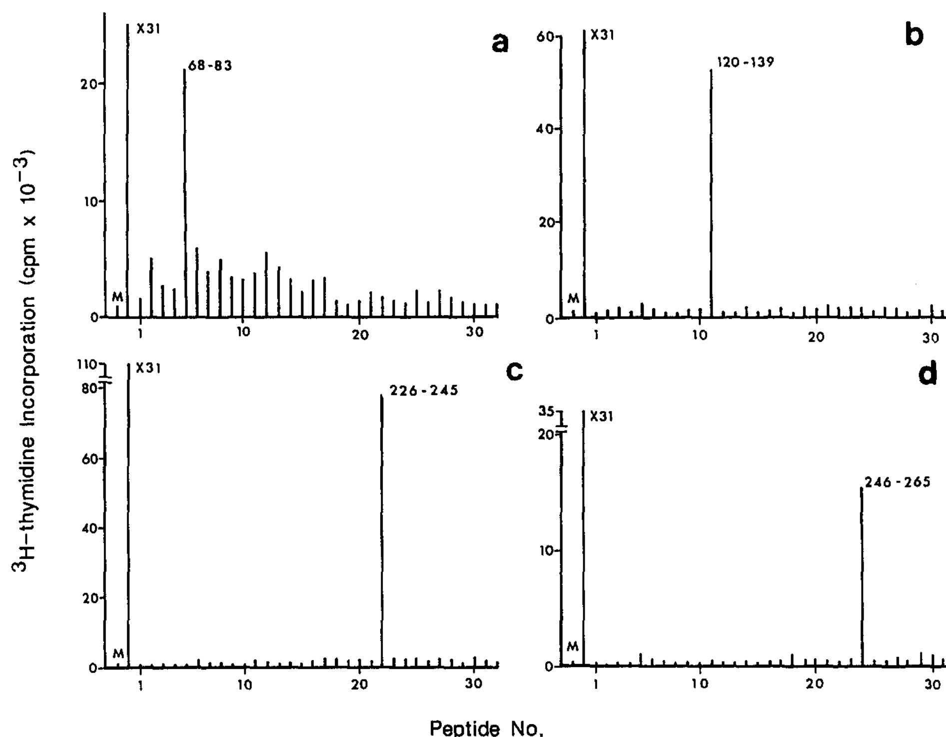

FIGURE 2. Proliferative responses of T cell clones against synthetic peptides corresponding to sequences within HA1 28–328. Results shown are from representative experiments for the mean [^{3}H]thymidine incorporation (cpm) for triplicate cultures, SE <10%, against each individual peptide, tested in a single assay at concentrations of, 1 μg/ml (clone 1.3 [*a*] and clone 1.99 [*d*]) and 0.5 μg/ml (clone 4.4.4 [*b*] and clone 3F10 [*c*]).

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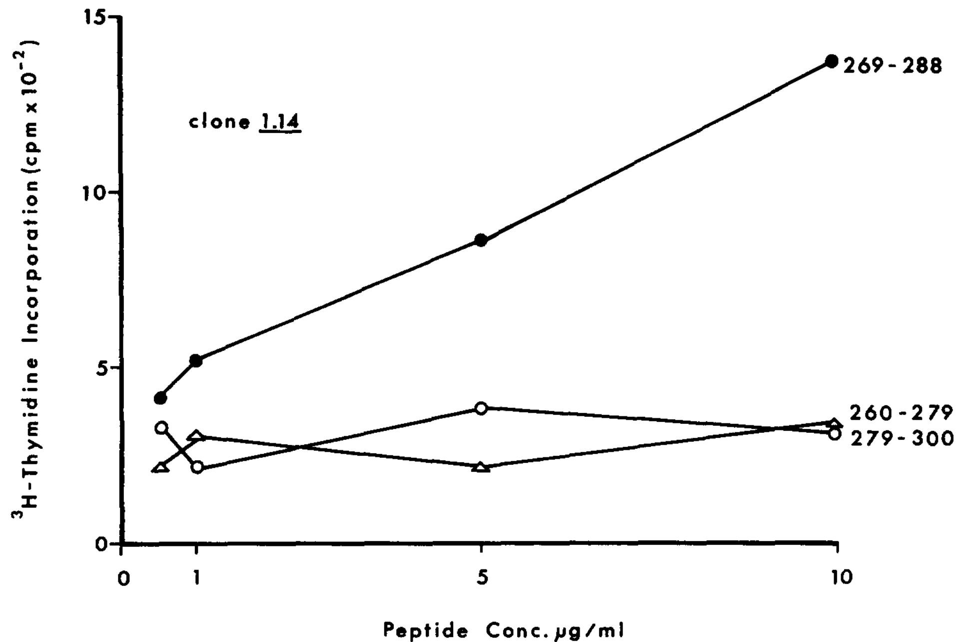

FIGURE 3. Recognition of HA1 peptide 269–288 by T cell clone 1.14. [^{3}H]Thymidine incorporation (cpm) for triplicate cultures.

in Fig. 3 for clone 1.14. T cell clones 1.3, 4.4.4, 3F10, and 1.99 gave similar results (data not shown), i.e., proliferating to their specific stimulatory peptides in the concentration range 0.1–5 µg/ml but not to adjacent flanking sequences.

MHC Restriction of T Cell Clones. Restriction analysis was performed on the T cell clones using splenic APCs from CBA, BALB/c, the B10.AQR recombinant that expresses H-2^k class II MHC alleles but only H-2^q and H-2^d class I alleles, and the recombinant mouse strain B10.4R which lacks the E^k_α chain and therefore can only utilize the I-A^k molecule for class II–restricted presentation of antigen. The results in Table I show that the HA-specific responses of each T cell clone were class II (Ia^k)-restricted since they could be stimulated by APC from both CBA and B10.AQR mice. Clones 1.3, 1.14, and 4.4.4 were I-A^k restricted since they could be stimulated by APC derived from CBA and B10.4R mice. Conversely, clones 3F10 and 1.99 recognized both virus and peptide in the context of I-E^k, since these clones were stimulated by APC from CBA, but not B10.4R mice. BALB/c-derived APC failed to present antigen to any of the T cell clones tested. Experiments using anti-Ia^k mAbs also supported these results (data not shown). These results suggest that peptides HA1 68–83, HA1 120–139, HA1 269–288, together with HA1 53–63 (14), are recognized by specific T cells in association with I-A^k; while HA1 226–245 and HA1 246–265 are presented in association with I-E^k.

Correlation between Peptide Specificities and Natural Variant Virus Reactivities of the T Cell Clones. The synthetic peptides had defined four I-A^k– and two I-E^k-restricted epitopes in the HA1 subunit and it was of interest to attempt to correlate the recognition specificity shown by individual clones for natural variants with drift substitu-

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TABLE I

MHC Restriction of T Cell Clones

Mouse strain	Antigen	[^{3}H]Thymidine incorporation				
		1.3	4.4.4	3F10	1.99	1.14
				cpm		
CBA	Medium	575	225	702	245	455
	X31	16,675	4,025	10,387	6,404	2,971
	Peptide	ND	20,009*	49,995‡	29,437§	1,858‖
B10/AQR	Medium	2,815	998	1,305	1,292	1,452
	X31	26,256	34,145	19,442	5,199	34,850
	Peptide	ND	18,394*	81,595‡	20,080§	3,648‖
B10/4R	Medium	298	269	420	465	216
	X31	13,245	3,067	170	237	1,703
	Peptide	ND	5,754*	174‡	500§	1,656‖
BALB/c	Medium	318	375	896	210	202
	X31	301	252	1,414	194	167
	Peptide	ND	284*	2,023‡	341§	123‖

Proliferative responses of T cell clones were measured in the presence of x-irradiated splenic APCs from various recombinant mouse strains against X31 virus (100 HAU/ml) or their specific HA1 synthetic peptides.

* 120–139 (1 μg/ml.

‡ 226–245 (1 μg/ml).

§ 246–265 (5 μg/ml).

‖ 269–288 (10 μg/ml).

ND = not determined.

tions that had occurred in the five antigenic regions (designated A–E [22]) of the HA1 subunit.

HA1 63–83: Antigenic Site E. Consider the drift substitutions that have occurred in this region of HA1 at residues 78 and 83 (antigenic site E of HA1 [22]). Natural variant viruses with these substitutions failed to stimulate optimally T cell clone 1.3 (Table II). Recognition of mutant viruses with a 78V → G change was reduced to <25% of that for the parental virus X31, while those with the 83T → K substitution, for example TEX/77, were nonstimulatory, suggesting that these drift residues are critical for class II-TCR recognition of antigen by clone 1.3.

HA1 120–139: Antigenic Site A. Table III demonstrates that T cell clone 4.4.4 failed to respond to mutant viruses HK/71 and BK/79. Unique amino acid drift substitutions occur at positions HA1 129 and 132 in HK/71, within the epitope 120–139, which is recognized by this clone and may be responsible for the inability of clone 4.4.4 to recognize this mutant. However, the basis of its nonresponsiveness to BK/79 is unclear at present. Proliferation of clone 4.4.4 was reduced by >60% in response to a laboratory mutant virus, 184, with a G → R substitution at HA1 135 (Table III). This substitution may render the sequence 118–138 susceptible to tryptic-like enzymatic cleavage, an assumption supported by the finding that the avian virus A/Duck/Ukraine/63, which also has a nonconservative substitution (G → E) at HA1 135, is nevertheless optimally recognized by clone 4.4.4 (Table III).

HA1 269–288: Antigenic Site C. Within the epitope HA1 269–288 recognized by clone 1.14, amino acid substitutions have occurred at HA1 residues 275, 278, and

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TABLE II
Correlation between Peptide Specificity and Natural Variant Reactivity of T Cell Clone 1.3

Virus	1.3 Reactivity	Amino acid sequence of reactive peptide															
		68		70								78			81		83
X31	+ +	D	A	L	L	G	D	P	H	C	D	V	F	Q	N	E	T
A/ENG/69	+ +														D		
A/HK/71	+ +																
A/ENG/72	+											G					
A/HAN/73	+											G					
A/PC/73	+											G					
A/SCO/74	+											G					
A/VIC/75	–											G					K
A/TX/77	–											G					K
A/BK/79	–											G					K
A/SOF/83	–											G					K
A/CN/84	–											G					K

T cell clones were tested at optimal viral concentrations (50–100 HAU/ml) against X31 virus and H3N2 natural variant viruses isolated between 1968 and 1984 with amino acid substitutions within their HA1 subunits. Responses were scored, in terms of the ability of virus to induce [^{3}H]thymidine incorporation to an equivalent level to that observed for X31. + +, 30–100% X31; +, 10–30%; –, <10% X31.

TABLE III
Correlation between Peptide Specificity and Natural Variant Reactivity of T Cell Clone 4.4.4

Virus	4.4.4 Reactivity	Amino acid sequence of reactive peptide																			
		120		122				126		128	129			132			135				139
X31	+ +	F	I	T	E	G	F	T	W	T	G	V	T	Q	N	G	G	S	N	A	C
A/ENG/69	+ +																				
A/HK/71	–										E			E							
A/ENG/72	+ +			N																	
A/HAN/73	+ +			N																	
A/PC/73	+ +			N																	
A/SCO/74	+ +			N																	
A/VIC/75	+ +			N				N											S		
A/TX/77	+ +			N				N											Y		
A/BK/79	–			N				N											Y		
A/SOF/83	+ +			N																	
A/CN/84	+ +			N				N							S				Y		
M1	+ +									N											
184	+																R				
DUCK/63	+ +																E		S		

See footnote to Table II.

279 (Table IV), which comprise an exposed loop within antigenic site C of HA (23). T cell clone 1.14 exhibited a decrease in its proliferative response to variants ENG/72, HN/73, PC/73, and SCO/74 by ~90% of that for X31 virus. This coincided with a D → G substitution at HA1 275 in these mutants. Natural variants with additional

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TABLE IV

Correlation between Peptide Specificity and Natural Variant Reactivity of T Cell Clone 1.14

Virus	1.14 Reactivity	Amino acid sequence of reactive peptide																			
		269						275			278	279									288
X31	+ +	R	S	D	A	P	I	D	T	C	I	S	E	C	I	T	P	N	G	S	I
A/ENG/69	+ +																				
A/HK/71	+ +																				
A/ENG/72	+							G													
A/HAN/73	+							G													
A/PC/73	+							G													
A/SCO/74	+							G													
A/VIC/75	–							G			S										
A/TX/77	–							G			S										
A/BK/79	–							G			S										
A/SOF/83	–										S	Y									
A/CN/84	–							G			S	Y									

See footnote to Table II.

changes at positions 278 and 279 were not recognized by this T cell clone. Therefore, the natural variant reactivities of clone 1.14 can be explained by the substitution of drift residues 275, 278, and/or 279 within its specific epitope.

HA1 226–245. Within the sequence HA1 226–245, amino acid substitutions have occurred at positions 226, 242, and 244 within natural variants, and 226, 227, and 228 within the avian virus A/Duck/Ukraine/63 (Table V). Clone 3F10 was insensitive to these drift substitutions that had occurred in this region, since this T cell clone responded to all natural variant viruses and also to the avian mutant. This

TABLE V

Correlation between Peptide Specificity and Natural Variant Reactivity of T Cell Clone 3F10

Virus	3F10 Reactivity	Amino acid sequence of reactive peptide																			
		226				230										240					245
X31	+ +	L	S	S	R	I	S	I	Y	W	T	I	V	K	P	G	D	V	L	V	I
A/ENG/69	+ +																				
A/HK/71	+ +																				
A/ENG/72	+ +																	I			
A/HAN/73	+ +	Q																I			
A/PC/73	+ +																	I			
A/SCO/74	+ +																	I		L	
A/VIC/75	+ +																	I			
A/TX/77	+ +																	I		L	
A/BK/79	+ +																	I		L	
A/SOF/83	+ +																				
A/CN/84	+ +																	I			
DUCK/63	+ +	Q	P	G																	

See footnote to Table II.

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TABLE VI

Correlation between Peptide Specificity and Natural Variant Reactivity of T Cell Clone 1.99

Virus	1.99 Reactivity	Amino acid sequence of reactive peptide																			
		246		248		250										260					265
X31	+ +	N	S	N	G	N	L	I	A	P	R	G	Y	F	K	M	R	T	G	L	S
A/ENG/69	+ +																				
A/HK/71	+ +																				
A/ENG/72	+ +																				
A/HAN/73	+ +																				
A/PC/73	+ +																				
A/SCO/74	+ +																				
A/VIC/75	+ +																				
A/TX/77	+ +															I					
A/BK/79	+ +															I					
A/SOF/83	+ +																				
A/CN/84	+ +			T												I					

See footnote to Table II.

suggests that the epitope seen by this clone lies within residues 229–241, a region buried within the HA tertiary structure (23) and whose primary sequence has been conserved throughout the natural variants tested. The finding that overlapping peptides 216–236 and 236–252 failed to stimulate clone 3F10 (Fig. 2 *c*) supports this proposal.

HA1 246–265. T cell clone 1.99 was also unable to differentiate between the natural variant viruses (Table VI). Within the specific sequence HA1 246–265, recognized by this clone, there have been two amino acid substitutions occurring in variant viruses tested, i.e., HA1 positions 248 and 260. The localization of the epitope seen by T cell clone 1.99 to within the conserved sequence 249–259 can explain natural variant crossreactivity of this clone.

T Cell Lines Recognize Immunodominant Epitopes. To compare the HA-specific T cell response at the population level with that exhibited by T cell clones, six T cell lines were established from individual CBA mice infected intranasally with X31 virus.

TABLE VII

Limited Recognition of Synthetic Peptides within HA1 28-328 by T Cell Lines Established from CBA Mice Infected with X31 Virus

	[^{3}H]Thymidine incorporation					
	CB1	CB3	CB4	CB7	CB9	CB11
	cpm					
Medium alone	200	150	200	350	350	250
X31	30,032	3,235	10,607	9,234	12,590	1,200
HA1 48-67	9,264	150	6,490	4,233	350	717
HA1 226-245	26,458	1,726	6,960	350	13,080	1,185

Cell lines were tested against virus (100 HAU/ml) or synthetic peptide (1 μg/ml) in T cell proliferative assays as described in Materials and Methods.

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After three restimulations in vitro with X31 and APC, the lines were screened, in T cell proliferation assays, against the panel of HA1 synthetic peptides. The lines responded to either HA1 48-67 or HA1 226-245, or both (Table VII), but not to the remaining peptides (data not shown). This indicates that a more limited T cell response is evident at the population level compared with that observed in extensive analysis at the clonal level for T cells of the same haplotype, and provides a cautionary note concerning immunodominance.

Discussion

In this study, the I-A^k and I-E^k-restricted $CD4^+$ T cell response against the HA1 subunit of influenza HA (H3N2) has been investigated using synthetic peptides corresponding to fragments of HA1 28-328 of X31 virus. At the population level, T cell lines focused upon two epitopes defined by synthetic peptides HA1 48-67 and HA1 226-245, with lines from individual mice showing proliferative responses to either or both peptides. In contrast, an extensive analysis of a panel of $CD4^+$ T cell clones revealed six distinct, nonoverlapping T cell epitopes within HA1 sequences 53-63/48-68 (13, 14), 68-83, 120-139, 269-288, restricted by the I-A^k molecule; and within HA1 226-245 and 246-265 recognized in the context of I-E^k. Furthermore, the complex pattern of recognition by these clones for natural variant viruses correlated with their specificities for peptides.

Estimates of diversity of the T cell repertoire based on in vitro T cell responses may be influenced by a number of factors, including the relative frequency of antigen-specific precursors, or the state of activation in the host prior to initiation of in vitro culture. Moreover, during the generation of T cell lines competition may occur between HA1 peptides having different relative affinities for binding to class II molecules (12), resulting in the preferential expansion of a population of cells specific for one or more immunodominant epitopes, as evident here in the response of T cell lines to HA1 48-67 and HA1 226-245. However, the significant findings of this study are that H-2^k-restricted, HA-specific T cell clones recognize several distinct regions of HA1 in the context of a single class II restriction element (I-A^k or I-E^k) and that the majority of clones are sensitive to amino acid substitutions in mutant viruses in regions of the HA1 subunit corresponding to B cell recognition sites.

In contrast to these findings, a consensus finding for murine $CD4^+$ T cell recognition of other antigens (e.g., references 1, 3, 5) has been to identify no more than one to three distinct T cell epitopes per mouse haplotype, resulting in the general conclusion that the expressed T cell repertoire exhibits limited diversity. Our present results demonstrate that, at least for the I-A^k-restricted response to HA, greater T cell diversity can be exhibited.

The class II-restricted recognition sites identified in this present study exhibit primary structural characteristics that have been used to predict the presence of T cell epitopes in other proteins. HA1 peptides 48-67 (14), 68-83, 120-139, and 226-245 contain regions with a high propensity for amphipathic α-helix formation (24), whereas peptides HA1 246-265 and 269-288 have poor amphipathicities (Berzofsky, J., personal communication). Furthermore, each sequence, apart from HA1 226-245, also has a 4- or 5-residue consensus motif, *Gly or charged; hydrophobic; hydrophobic; polar (or hydrophobic, polar)*, shown to be common to the majority of other T cell epitopes by Rothbard and Taylor (25). However, there appears to be no obvious sequence

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homology or other common structural feature shared by those T cell epitopes on HA restricted to either I-E^k or I-A^k. Recent studies have implicated a single binding site for peptides within class I (26) and class II (12, 27) MHC molecules, thereby suggesting that peptides restricted to identical MHC alleles may share similar structural characteristics. MHC-linked similarities within the six HA1 peptides studied here may be more apparent when the minimum stimulatory peptide lengths have been determined. Alternatively, the lack of a common feature among HA1 T cell epitopes sharing common class II restriction may suggest that Ia molecules possess a greater capacity to bind structurally dissimilar peptides than has been generally assumed.

Extensive sequence analysis of H3N2 natural variant viruses isolated between 1968–1984 and mAb-selected laboratory mutants have identified five antigenic sites within HA1, where accumulated amino acid drift substitutions are clustered (22). HA-specific T cell clones from two of six specificity groups analyzed in this present study failed to discriminate between natural variants and laboratory mutants. These clones recognized epitopes within HA1 226–245 and 246–265. Both regions contain substantial sequences of amino acids that have been conserved in the variant viruses tested (i.e., HA1 229–241 and 249–259). The inability of peptides overlapping and flanking these epitopes to stimulate the relevant T cell clones suggests that the clones specific for these sequences recognize areas within the conserved regions, a conclusion that would account for their failure to discriminate between the natural variants tested. However, the majority of $CD4^+$ clones were sensitive to amino acid drift substitutions. A close correlation was observed between greatly reduced, or nonrecognition of natural variant viruses by T cell clones 1.3., 4.4.4, and 1.14, and amino acid substiutions at HA1 positions 78/83, 275/278, and 129/132 within their specific T cell epitopes.

Amino acid changes at these positions may be affecting the processing of the HA molecule or alternatively, class II-TCR interaction. Recent reports have shown that amino acid substitutions within proteins such as myoglobin (28) or cytochrome (29) can affect the processing and presentation of specific T cell epitopes. Also, class II-TCR contact residues on T cell epitopes have been identified using truncated and substituted peptides (30–32). Such investigations have shown that class II and TCR contact residues may either be segregated within a T cell recognition site (31), or that a single amino acid residue may be involved in both Ia and TCR interaction (32). In the present study, amino acids within the HA1 subunit of H3N2 influenza virus that have undergone substitution in natural variants have defined residues that affect recognition by I-A^k-restricted T cell clones. Further analysis of these drift residues by site-directed mutagenesis of the cloned HA gene and by their corresponding substitution in synthetic peptide analogues may determine whether they are residues critical for processing or for interaction with Ia or TCR. Such use of peptide analogues has tentatively identified residue HA1 54N as a TCR contact site for a group of T cell clones that recognize synthetic peptide HA1 53–63 and that are sensitive to the drift substitution 54N → S in natural variant viruses (14).

Other studies have also described drift-sensitive, class II–restricted T cell clones specific for HA. For example, H-2^d-restricted clones that react with HA1 (H1N1) 109–119 and 126–138 identified by Hurwitz and colleagues (33) failed to respond to laboratory mutant viruses with single amino acid substitutions at positions 115 (E

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→ K) and 136 (S → P), respectively, within the HA1 subunit. Also, the majority of HA-specific T cell clones established by Brown et al. (34) were sensitive to amino acid drift substitutions within HA and HA1 positions 60 and 63.

Finally, an immediate question that arises from our current finding is the mechanism and significance of the high frequency of HA-specific T cells observed that are sensitive to amino acid substitutions within the HA1 subunit that occur during antigenic drift. This association may imply that MHC polymorphism or TCR repertoire diversity are contributing factors in the immune pressure for antigenic drift in influenza HA. It is generally assumed that the primary determinant of antigenic drift is the neutralizing antibody response since laboratory mutant viruses selected with mAbs have sequence changes within their HA molecules that have also featured in natural variants (22). Moreover, it is difficult to envisage a selection mechanism for mutant viruses operating at the TCR level and there have been conflicting reports concerning the influence of MHC restriction on the antibody response to HA (35, 36). Whatever the structural basis for the commonality of the class II-restricted repertoire and the neutralizing antibody response to major antigenic sites of the HA1 subunit, the sensitivity of $CD4^+$ clones to substitutions that arise during antigenic drift may be an important factor in the ability of mutant influenza viruses to evade immune recognition.

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Summary

$H\text{-}2^k$-restricted T cell clones derived from CBA mice infected with X31 (H3N2) influenza virus, were shown to recognize distinct, nonoverlapping sequences within the HA1 subunit of the viral hemagglutinin (HA) using synthetic peptides. Three $I\text{-}A^k$-restricted T cell sequences were identified within HA1 68–83, 120–139, and 269–288, and two recognition sites presented in the context of the $I\text{-}E^k$ molecule were mapped to HA1 sequences 226–245 and 246–265. T cell clones specific for these regions of HA1 demonstrated varying abilities to differentiate between natural variant viruses that had accumulated substitutions within their HA molecules as a result of antigenic drift. Clones that recognized sequences HA1 226–245 and HA1 246–265 failed to discriminate between natural variants and focused on conserved sequences within these epitopes. A majority of T cell clones were sensitive to amino acid substitutions that have featured in antigenic drift occurring within three major antigenic sites of the HA1 subunit; substitutions at HA1 residues 78 (V)/83(K) and 275(D)/278(I) within the HA1 subunit of mutant viruses correlated with a 75% reduction in the proliferative response for T cell clones specific for sequences HA1 68–83 and HA1 269–288, respectively. Furthermore, a clone that recognized HA1 120–139 was nonresponsive to a mutant virus HK/71, implicating amino acids at HA1 position 129(G) and/or 132(Q) within this sequence as crucial for recognition.

Our data, together with the previous finding that sequence HA1 53–63 is also a major $I\text{-}A^k$-restricted T cell recognition site, demonstrate a level of diversity in the T cell recognition of influenza HA, within a single mouse haplotype hitherto unrecognized, and imply that the T cell repertoire diversity against foreign antigens may be greater than previously assumed. Furthermore, the frequency at which HA-specific T cells have been identified that focus on amino acids within the HA1 subunit of HA also featuring in antigenic drift, suggests that a failure of MHC class II-restricted T cells to recognize specific epitopes within mutant HA molecules may con-

tribute significantly to the capacity of variant influenza viruses to evade immune recognition.

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