

Distinct Epitopes Recognized by $I-A^d$ -Restricted T-Cell Clones within Antigenic Site E on Influenza Virus Hemagglutinin

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A total of 14 $I-A^d$ -restricted helper T-cell clones specific for the hemagglutinin (HA) molecule of influenza virus were isolated from spleens of BALB/c or (BALB/c × C57BL/10)F₁ mice immunized with the H3 subtype influenza virus A/Memphis/71 (Mem 71) and from lymph nodes of BALB/c mice primed with purified HA. The specificity of these T-cell clones was assessed in proliferation assays by reactivity with naturally occurring strains of viruses that arose by antigenic drift and contain known amino acid sequence changes in HA and with a panel of monoclonal antibody (MAb)-selected mutants of Mem 71 with single amino acid substitutions in HA. The HA genes of those mutant viruses that failed to stimulate one or more of the T-cell clones were sequenced. The clones could be allocated to at least four groups, each group having a distinct pattern of reactivity with the panel of natural field strains. The epitopes recognized by the four groups of clones were found, by reactivity with MAb-selected mutants, to be in very close proximity to one another and probably overlapping. All of the distinct epitopes recognized by the T-cell clones were adversely affected by a single amino acid substitution, either at residue 60 or at residue 63 in the HA₁ polypeptide chain, within the region known from antibody-binding studies as site E. Some, but not all, of the epitopes may be influenced by the addition of a carbohydrate side chain to the HA of a particular MAb-selected mutant and certain field strains containing an Asp → Asn substitution at residue 63. Site E is therefore a major site of $H-2^d$ helper T-cell recognition on the H3 HA.

Much is now known about the nature, number, and distribution of the epitopes on the influenza viral hemagglutinin (HA) molecule to which neutralizing antibodies bind (35). Analysis of the ability of monoclonal antibodies (MAbs) to bind to field strains that have arisen sequentially in nature by antigenic drift, or to laboratory mutants selected by growth in the presence of a particular MAb (12), has revealed the existence of up to hundreds of distinguishable epitopes (13, 29, 32) which tend to be concentrated around five major antigenic regions (sites) on the accessible surface of the HA molecule (7, 36).

More recently, the epitopes on the HA molecule that are recognized by helper T (T_H) cells have come under investigation. In an early study with uncloned T-cell populations, Anders et al. (1) demonstrated that T_H cells directed to HA respond to a wider range of influenza viral strains than bind anti-HA antibodies. Lamb et al. (19) then demonstrated that immune T cells could respond to synthetic peptides corresponding to regions located within the interior of the HA as well on its surface. It was also shown that the HA₂ chain of the molecule or peptides from it could restimulate T cells from virus-primed mice (2, 17). Together, these studies revealed that the epitopes recognized by HA-specific T cells include some in relatively conserved regions of the molecule not recognized by antibodies or B cells.

Subsequent studies with cultured T-cell lines and clones have been able to locate more precisely the regions of the HA with which T_H cells interact. The majority of these defined regions could be represented by short synthetic peptides comprising part of the linear sequence of the HA₁

chain. Such peptides presumably contain not only the amino acid residues required for interaction with the T-cell receptor (the epitope) but also those required for interaction with the class II major histocompatibility complex (MHC) antigen used to present that peptide (the agretope) postulated by Schwartz (27). These peptides have been found by screening clones with mutant viruses having single amino acid changes in the HA and then synthesizing the region encompassing any residue that could not be substituted without a loss of reactivity. Alternatively, synthetic peptides or cyanogen bromide-derived fragments have been empirically screened.

Using these approaches, Hackett et al. (14) and Hurwitz et al. (16) have defined three major sites of recognition by BALB/c ($H-2^d$) T_H cells on PR8 (H1) HA. While site 1 (residues 111 to 119) and site 2 (residues 126 to 138) are in regions of the PR8 HA molecule that are also recognized by antibody, site 3 (residues 302 to 313) comprises a relatively conserved sequence of amino acids near the C terminus of HA₁ which is not recognized by antiviral antibody. Likewise, Lamb and Green (20) found that the T_H clones they isolated from humans with a history of exposure to H3 influenza virus responded either to a synthetic peptide encompassing the same C-terminal region of the HA₁ of this subtype or to a large synthetic peptide representing the region equivalent to sites 1 and 2 on the PR8 HA. A panel of murine $I-A^k$ -restricted clones described by Thomas et al. (31) was also found to recognize a peptide from X-31 (H3) HA (residues 118 to 138) which occupied a surface site that included the equivalent of site 1 of the H1 HA.

Together, these studies with viruses of two subtypes and T cells from humans as well as from two strains of mice indicate that these three regions of the HA molecule contain

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important sites of recognition for T cells. Recently, Mills et al. (23) have described at least two additional sites recognized by HA-specific T-cell clones obtained from CBA (*H-2^b*) mice infected with X-31 (H3) virus. Some of these 27 clones recognized a synthetic peptide (residues 48 to 68) encompassing sites C and E of the H3 HA, whereas others appeared to recognize an epitope that was dependent on conformational integrity of the HA, situated around residue 208 at the interface between monomers of the HA trimer. While the sites recognized by the remaining clones were not defined, the fact that they could be placed into several different groups on the basis of their pattern of recognition of a panel of H3 field strains was interpreted as revealing a greater diversity in the T_H cell repertoire than had previously been recognized for influenza HA (16, 20) or for nonviral proteins (4). However, the differences in the reported number of T-cell recognition sites on HA may be more apparent than real. As with epitopes recognized by MAbs, there may be a limited number of antigenic regions (sites) but each site may contain a substantial number of overlapping epitopes which can be distinguished by different T-cell clones. The data presented in this report support this hypothesis. We demonstrate that site E on the HA molecule of the A/Memphis/71 (Mem 71) strain of H3 influenza virus represents a major site for recognition by *H-2^d*-restricted T cells but that different clones can discriminate among at least four overlapping epitopes involving amino acids Asp-60 or Asp-63 and nearby residues.

MATERIALS AND METHODS

Viruses. The following strains of influenza A virus of the H3N2 subtype were used, some in the form of recombinants with A/Bellamy/42 and bearing the H3 HA and N1 neuraminidase: A/Duck/Ukraine/1/63 \times A/Bellamy/42 (Duck 63); A/England/878/69 (Eng 69); A/Memphis/1/71 \times A/Bellamy/42 (Mem 71); A/Memphis/102/72 \times A/Bellamy/42 (Mem 72); A/Port Chalmers/1/73 (PC 73); A/Victoria/3/75 (Vic 75); A/Papua New Guinea/75 \times A/Bellamy/42 (PNG 75); A/Texas/1/77 (Tex 77); A/Bangkok/79 (Bang 79); and A/Philippines/2/82 (Phil 82). A/Japan/305/57 \times A/Bellamy/42 (Jap 57) of the H2 subtype and the influenza B virus B/Lee were also used.

MAB-selected mutants (escape mutants) were isolated by growth of Mem 71 virus in the presence of neutralizing MAB to HA as described by Gerhard and Webster (12). The Mem 71 variants JANE, DON, TED, KEN, DOUG, and IAN were a gift from W. G. Laver.

Viruses were grown for 2 days in the allantoic cavity of 10-day embryonated hen eggs. Allantoic fluid containing virus was stored in aliquots at -70°C and was used for immunization of mice, stimulation of T-cell clones in vitro, and proliferation assays. Viruses used for nucleic acid sequencing were concentrated from allantoic fluid either by use of a Diaflo hollow-fiber system with H1-P100-43 cartridge (Amicon Corp., Lexington, Mass.) or precipitation with 8% (wt/vol) polyethylene glycol 6000 (BDH Chemicals, Australia) and purified by rate-zonal sedimentation on 25 to 70% sucrose gradients (3).

Purified HA. Isolated Mem 71 HA was prepared from purified virus by bromelain (Sigma Chemical Co., St. Louis, Mo.) digestion (5). Briefly, virus was digested (18 h in 0.1 M Tris hydrochloride [pH 8.0], 0.05 M 2-mercaptoethanol) at an enzyme-to-viral-protein ratio of 1:2. Virus cores were spun out in a Beckman air-driven centrifuge (100,000 $\times g$, 10 min). The soluble bromelain HA was then purified over a 5 to

20% sucrose gradient in phosphate-buffered saline (pH 7.4) containing 0.1% sodium azide for 16 h at 40,000 rpm in a Beckman SW41 rotor.

Mice. Inbred female 6- to 8-week-old BALB/c, (BALB/c \times C57BL/10)F₁, B10.A, and D2.GD mice, bred in our animal facility, were used as a source of immune T cells or of antigen-presenting cells (APC) in culture.

Culture medium. The culture medium was RPMI 1640 (Flow Laboratories, McLean, Va.) supplemented with 10% fetal calf serum (heat inactivated at 56°C for 30 min), 2 mM L-glutamine, 2 mM pyruvate, 0.1 mM 2-mercaptoethanol, and 30 μg of gentamicin per ml.

Preparation and maintenance of T-cell clones. The method used to establish T-cell lines was based on that of Gerhard et al. (11). Individual BALB/c and (BALB/c \times C57BL/10)F₁ mice were injected intraperitoneally with a single dose of 400 hemagglutinating units (HAU) of infectious influenza virus, and 12 days later the spleens were removed. An alternative source of immune T cells was a pool of inguinal and popliteal lymph nodes from seven BALB/c mice which had been injected 7 days previously in the footpads with 10 μg of purified HA in complete Freund adjuvant. Spleen cells or lymph node cells ($5 \times 10^6/\text{ml}$) were cultured in plastic 75-cm³ flasks (Nunc, Roskilde, Denmark) for 5 days in medium containing 30 HAU of Mem 71 virus per ml. The T cells, passaged at 2×10^5 cells per ml, were maintained in long-term culture by stimulation with Mem 71 virus (30 HAU/ml) in the presence of 2×10^6 gamma-irradiated (2,200 R) normal BALB/c or (BALB/c \times C57BL/10)F₁ spleen cells per ml as a source of APC for 4 to 5 days at a time, alternating with 7 days in medium containing interleukin-2 (IL-2). The source of IL-2 was a crude supernatant from BALB/c spleen cells cultured at 10^7 cells per ml for 42 h with 5 μg of concanavalin A per ml and was used at a final concentration of 5%. After approximately 4 weeks, T-cell clones were derived from these lines by limit dilution as previously described (L. E. Brown, J. M. Katz, R. A. Ffrench, E. M. Anders, and D. O. White, Cell. Immunol., in press). Briefly, wells of 96-well plates were seeded with T cells and 10^6 APC in medium containing 60 HAU of virus and approximately 200 IU of IL-2. Clones were picked only from wells in which the T-cell number used yielded less than 30% of replicate wells positive for growth.

Proliferation assay. Before being used in culture, T-cell clones were depleted of antigen and dead APC by passage over an Isopaque-Ficoll gradient (8). Microcultures were established in triplicate in 96-well flat-bottomed tissue-culture plates (Nunc) containing 10^4 T cells, 3×10^5 APC, and various doses of infectious influenza virus or purified HA in a total volume of 0.25 ml. Plates were incubated at 37°C in 5% CO₂ for 4 days with addition of 0.5 μCi of [³H]thymidine in the final 18 h. Cells were then harvested onto glass-fiber filters for liquid scintillation counting.

MAbs. MAbs 36, 244, and 207, specific for the HA of Mem 71 virus, were raised by L. E. Brown and J. M. Murray. All other HA-specific MAbs used for the selection of variants were prepared by P. A. Underwood; the specificity of different groups of these MAbs has been described elsewhere (32). For reference to that report, MAbs A83 and A195 belong to group 1; MAb A170 belongs to group 2; MAbs A67, A127, A152 and A193 belong to group 3; MAb A63 belongs to group 4; MAb A200 belongs to group 5; MAbs A45 and A212 belong to group 6; MAbs A205 and A183 belong to group 8; MAbs A221 and A218 belong to group 9; MAbs A95 and A106 belong to group 10; and MAbs A119 and A158 are unclassified.

Ascitic fluids containing MAbs to *H-2^d* gene products were used in blocking assays. Hybridoma cell lines 14-4-4 (anti-I-E^{k,d}, specificity Ia.7) (25) and 35-1-2S (anti-K^dD^d) (26) were obtained from I. F. C. McKenzie (University of Melbourne) and N. Koch (Walter and Eliza Hall Institute, Melbourne), respectively. MAb 34-5-3S (anti-I-A^d) (26) was purchased as ascitic fluid from Litton Bionetics (Charleston, S.C.).

Hemagglutination and hemagglutination inhibition assays. Hemagglutination and hemagglutination inhibition assays were performed according to the method of Fazekas de St. Groth and Webster (10).

Sequencing the HA gene. RNA was extracted from preparations of purified virus by using sodium dodecyl sulfate-proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation. Synthetic DNA oligomers complementary to nucleotides 13 to 27, 201 to 215, 411 to 425, 597 to 611, 798 to 812, and 945 to 959 of Mem 71 viral HA RNA were used as sequencing primers in dideoxy chain-termination reactions. The sequencing protocol was essentially the same as described by Naeve et al. (24), except that [³⁵S]dATP was used instead of [³²P]dATP.

RESULTS

HA-specific T-cell clones. T-cell lines were prepared from spleen cells of a BALB/c and a (BALB/c × C57BL/10)F₁ mouse immunized with Mem 71 influenza virus and also from lymph node cells of BALB/c mice immunized with purified Mem 71 HA. The lines were maintained in vitro by restimulation with Mem 71 virus and APC. T-cell clones were derived from them by limit dilution. Of the clones obtained from the (BALB/c × C57BL/10)F₁ mouse, only those responding to antigen on APC of the BALB/c (*H-2^d*) parental type, which represented the great majority, are included in this report. Table 1 shows the origin of each of 14 T-cell clones and their ability to respond to virus and HA in proliferation assays. All of the clones were found to be stimulated by the homologous virus, Mem 71 (H3), and also by HA isolated from this strain. They did not recognize type A influenza virus of a different HA subtype (Jap 57) or a type B virus (B/Lee).

Phenotype and genetic restriction of T-cell clones. The method of derivation of T-cell clones used for this study is

known to favor clones of the L3T4⁺ (helper) phenotype (11; Brown et al., in press) which recognize antigen in association with the class II MHC molecules I-A and I-E. Representative clones were confirmed to be L3T4⁺ by cytotoxicity tests with anti-L3T4⁺ MAb GK1.5 (9) and complement (data not shown).

To determine the particular MHC molecule that serves as the restriction element for each clone, the clones were tested for their proliferative response to Mem 71 virus presented by APC from recombinant mouse strains. The data for clone F1-36 are shown as an example in Table 2. Despite the different origins of these *H-2^d*-restricted clones, in every case the T cells responded when virus was presented by APC from D2.GD mice, which bear the *d* allele at *K*, *A_α*, and *A_β* only. The clones did not respond to APC from mice of the B10.A strain, which have the *d* allele at the *D* locus only. This indicates that every clone described in this study is restricted in its recognition of viral antigen by genes in the region encoding *K* or *I-A*. The restriction element was confirmed to be *I-A^d* rather than *K^d* by demonstrating that the proliferative response to virus presented by BALB/c APC was inhibited in the presence of MAb against *I-A^d* but not by antibody against the other *H-2^d* gene products (Table 2).

Antigen specificity of T-cell clones. To determine the different T-cell specificities elicited by the H3 HA in this study, each clone was tested for reactivity with a panel of influenza A virus strains which have arisen by the accumulation of mutations in the HA gene during the process of antigenic drift (35) in the H3 subtype. In addition to nine human virus strains first isolated between 1969 and 1982, the avian H3 virus, Duck 63, was also examined, as its HA gene shows a high degree of homology with that of the human H3 subtype and is thought to be its progenitor (35). The proliferative response of the 14 clones to each virus is shown in Table 3. Comparison of the reactivity patterns revealed that the clones could be assigned to different groups. The T-cell clone derived from the virus-primed BALB/c mouse had a unique reactivity pattern, while the clones derived from F1 mice fell into at least two distinct groups, and the clones from HA-primed mice formed an additional group.

The different reactivity patterns can be more easily compared in Fig. 1, in which a representative T-cell clone from

TABLE 1. Proliferation of T-cell clones in response to influenza virus or HA

T-cell clone	In vivo origin		In vitro proliferative response ^a to:				
	Mouse strain	Antigen ^b	Mem 71 virus (type A, subtype H3)	Jap 57 virus (type A, subtype H2)	Lee virus (type B)	Mem 71 HA	No antigen
Mem clone 1	BALB/c	Virus	30,195	29	128	14,892	324
F1-5	F1 ^c	Virus	16,170	33	39	8,326	238
F1-33	F1	Virus	22,017	87	16	8,978	39
F1-36	F1	Virus	25,861	62	101	24,714	1,040
F1-40	F1	Virus	24,869	17	68	23,830	276
F1-48	F1	Virus	50,347	16	67	26,846	217
F1-51	F1	Virus	2,737	23	9	2,223	17
F1-52	F1	Virus	46,247	136	28	27,009	54
F1-55	F1	Virus	10,820	109	27	9,175	67
F1-70	F1	Virus	4,017	26	29	1,217	44
HA-1	BALB/c	HA	12,866	82	245	4,959	255
HA-3	BALB/c	HA	95,568	1	37	54,964	25
HA-7	BALB/c	HA	4,435	90	388	3,535	281
HA-11	BALB/c	HA	30,219	69	273	26,013	272

^a Data represent the mean of the thymidine incorporation (cpm) of triplicate cultures stimulated with either 100 HAU of virus or 2 µg of purified HA.

^b T-cell clones were derived from the spleens of mice primed with 400 HAU of infectious Mem 71 virus or from the lymph nodes of mice primed in the footpads with 10 µg of purified Mem 71 HA in complete Freund adjuvant.

^c (BALB/c × C57BL/10)F₁.

TABLE 2. Genetic restriction of T-cell clones

APC ^a	MAb ^b	% Proliferative response of T-cell clones ^c	
		F1-36	V5/1 ^d
BALB/c		100.0	100.0
B10.A		4.9	0.1
D2.GD		163.2	0.2
BALB/c	Anti-I-A ^d	7.3	92.3
BALB/c	Anti-I-E ^d	147.7	0.7
BALB/c	Anti-K ^d D ^d	111.2	100.6

^a The APC used have the alleles at *K I-A_α I-A_β I-E_α I-E_β D* shown in parentheses as given by Klein et al. (18): BALB/c (ddddd); B10.A (kkkkk); D2.GD (ddd^dbb).

^b MAbs anti-I-A^d (34-5-3S), anti-I-E^d (14-4-4), or anti-K^dD^d (34-1-2), when added, were present throughout the culture period at a 10⁻² final dilution.

^c Stimulation index was calculated as (mean cpm incorporated in triplicate cultures containing 100 HAU of Mem 71 virus)/(cpm incorporated in the absence of antigen). Results are expressed as a percentage of the stimulation index obtained with BALB/c APC in the absence of antibody. Background incorporation in the absence of antigen with BALB/c, B10.A, and D2.GD APC was 76, 65, and 268 cpm, respectively, for clone F1-36, and 93, 185, and 79 cpm, respectively, for clone V5/1.

^d V5/1 is an I-E^d-restricted, peptide-specific T-cell clone unrelated to those described in this study but cross-reactive with Mem 71 virus and was included as a control for antibody activity and specificity in this experiment.

each group is shown. Mem clone 1, which forms group 1, reacts with all strains up to and including the 1973 isolate, although its response to Mem 72 and PC 73 is lower than that observed for the earlier strains. Group 2 clones, comprising F1-40, F1-33, and F1-70, react only with Duck 63, Mem 71, and Mem 72. The clones in group 3 are stimulated strongly by Mem 71 and Mem 72 and to a lesser extent by all five of the other strains isolated before 1976. This group has been tentatively divided into two subgroups which may be recognizing subtly different epitopes; group 3b clones (F1-48, F1-55, and F1-36) respond well to all viruses up to and including PNG 75, while group 3a clones (F1-51, F1-52, and F1-5) do not respond as strongly to the five additional strains. Group 4 clones, derived from HA-primed mice, react with all the H3 strains tested.

Definition of epitopes recognized by T-cell clones. Comparison of the published amino acid sequences (33) of the HA from these naturally occurring strains of virus provides an indication of residues that may be important in recognition of HA by any particular T-cell clone. Often, however, there are several changes in different regions of the molecule that could account for the pattern of responses, and it is not

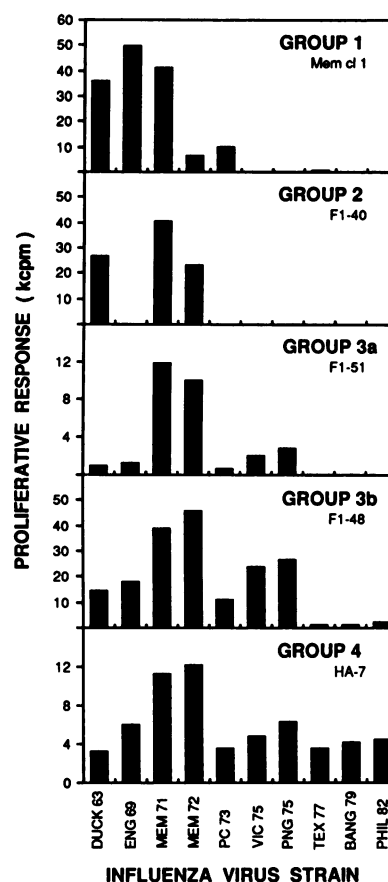


FIG. 1. Comparison of the reactivity of T-cell clones representative of each group with strains of influenza A virus of the H3 subtype. Data are expressed as (mean cpm of thymidine incorporated in the presence of antigen) minus (mean cpm in the absence of antigen).

possible to differentiate among these alternatives. Reactivity of the T cells with a panel of MAB-selected mutants was therefore investigated. Such escape mutants usually differ from the parent virus by only a single amino acid substitution in the HA (21); thus any difference in stimulatory capacity of a mutant compared with that of the parent virus can be attributed directly to a particular amino acid change.

A large panel of escape mutants was selected from the

TABLE 3. Reactivity of T-cell clones with strains of influenza A virus of the H3 subtype

Virus	% Proliferation of T-cell clones ^a													
	Mem clone 1 (1)	F1-40 (2)	F1-33 (2)	F1-70 (2)	F1-51 (3a)	F1-52 (3a)	F1-5 (3a)	F1-48 (3b)	F1-55 (3b)	F1-36 (3b)	HA-1 (4)	HA-3 (4)	HA-7 (4)	HA-11 (4)
Duck 63	87.9	66.1	77.9	36.7	7.9	2.5	12.3	36.8	6.7	24.7	27.3	16.0	29.3	46.8
Eng 69	120.9	0.3	2.1	14.0	10.3	4.7	13.9	45.4	23.1	11.7	63.9	36.0	53.1	45.5
Mem 71	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Mem 72	15.9	57.4	116.3	35.8	84.6	35.1	52.6	117.7	157.6	206.9	169.4	192.9	107.7	117.7
PC 73	24.1	0.0	0.3	0.0	5.4	0.1	0.9	28.1	20.0	16.9	38.3	17.8	31.7	36.0
Vic 75	0.0	0.0	1.0	0.0	16.8	6.5	18.7	61.2	19.4	44.5	49.8	22.8	41.9	41.9
PNG 75	0.0	0.0	0.3	0.0	23.0	4.8	28.5	68.8	30.3	67.7	83.7	76.7	55.6	63.1
Tex 77	0.9	0.9	0.9	0.0	0.0	0.0	0.6	3.2	0.2	0.7	28.0	4.3	31.7	18.4
Bang 79	0.5	0.0	0.0	0.0	0.0	0.7	2.7	3.5	1.4	0.5	32.9	10.9	36.8	34.0
Phil 82	0.5	0.0	0.1	0.0	0.0	0.0	1.7	5.4	0.7	2.1	32.9	9.7	40.3	40.7

^a Data represent mean of thymidine incorporation (cpm) in triplicate cultures stimulated with 10 HAU of virus minus the mean cpm of triplicate cultures containing no antigen, expressed as a percentage of the value obtained with Mem 71 virus. Numbers in parentheses indicate T-cell groups.

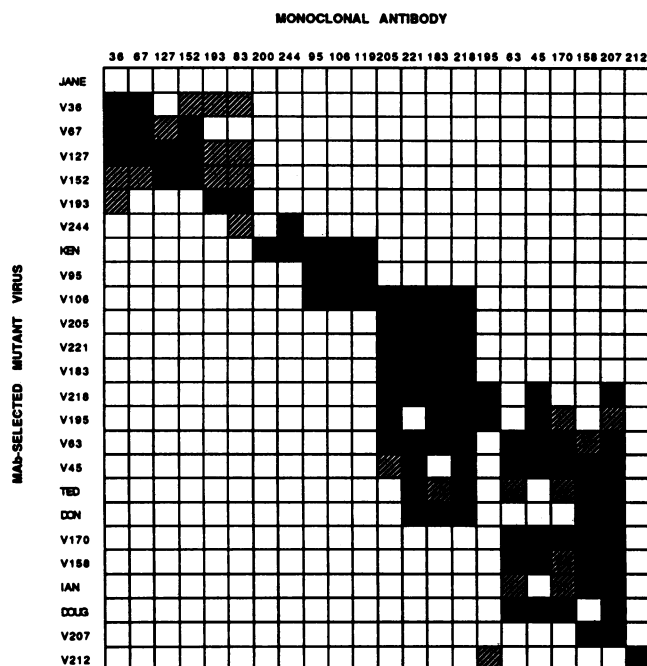


FIG. 2. Antigenic relationship between escape mutants of Mem 71 selected with MAbs against the H3 HA. Each MAb was tested for its ability to inhibit the hemagglutination of each of the mutant viruses and the parent virus Mem 71. ■, >16-fold decrease in the HI titer of the MAb against the mutant compared with the parent; ▨, 4- to 16-fold difference; □, <4-fold difference. Mutants with known amino acid substitutions in the HA are JANE, Asn-53 → Lys (site C); KEN, Pro-143 → Thr (site A); TED, Gly-218 → Trp (site D); DON, Ser-205 → Tyr (site D); IAN, Asn-188 → Asp (site B); and DOUG, Gln-189 → His (site B). The HA genes of V67, V127, and V152 were sequenced for this study.

parent Mem 71 virus by growth in the presence of MAbs specific for the H3 HA. These mutant viruses, together with six others obtained from W. G. Laver in which the amino acid substitutions in the HA are known, were tested in hemagglutination inhibition assays against each of the MAbs to determine their antigenic relationships (Fig. 2). It is clear that the great majority of escape mutants display a unique antigenic profile and thus presumably have a unique amino acid substitution. In this type of analysis, a grouping of antibodies and viruses can be achieved, each group being thought to define overlapping epitopes within a particular antigenic site (13, 34). Mutant viruses with changes in each of the known major antigenic areas on the HA (7, 32, 36) are represented in the panel.

Table 4 summarizes the ability of each of these escape mutants to stimulate proliferation of the clones representative of each T-cell group. Each clone responded to the majority of the mutant viruses but had decreased reactivity with V67, V127, and/or V152. Since V67, V127, and V152 have mutations that affect the same antigenic site, as determined by reactivity with the MAb panel in Fig. 2, the distinct epitopes recognized by the four groups of T-cell clones (Fig. 1) appear to be situated in the same region of the HA molecule.

To determine whether the lack of response to particular escape mutants shown by the representative T-cell clones (Table 4) was true for every member of the same T-cell group, all clones were tested for their ability to be stimulated by V67, V127, V152, and also by V36 which, from antibody

TABLE 4. Ability of MAb-selected mutants to stimulate proliferation of T-cell clones

Virus ^a	Proliferative response of representative T-cell clones				
	Group 1 Mem clone 1	Group 2 F1-40	Group 3a F1-51	Group 3b F1-48	Group 4 HA-1
Mem 71	+ ^b	+	+	+	+
JANE	+	+	+	+	+
V36	+	+	+	+	+
V67	—	+	+	+	+
V127	+	—	—	—	—
V152	+	+	+	+	—
V193	+	+	+	+	+
V224	+	+	+	+	+
KEN	+	+	+	+	+
V95	+	+	+	+	+
V106	+	+	+	+	+
V205	+	+	+	+	+
V221	+	+	+	+	+
V183	+	+	+	+	+
V218	+	+	+	+	+
V195	+	+	+	+	+
V63	+	+	+	+	+
V45	+	+	+	+	+
TED	+	+	+	+	+
DON	+	+	+	+	+
V170	+	+	+	+	+
V158	+	+	+	+	+
IAN	+	+	+	+	+
DOUG	+	+	+	+	+
V207	+	+	+	+	+
V212	+	+	+	+	+

^a Cultures were stimulated with 10 HAU of virus.

^b +, >40% of proliferative response to Mem 71; —, 0 to 25% of proliferative response to Mem 71.

analysis (Fig. 2), apparently has a mutation affecting this same antigenic site. Table 5 shows that all members of any given T-cell group behaved similarly to one another in their response to these variants. The amino acid substitution in

TABLE 5. Proliferative response of each T-cell clone to antigenically related mutants V36, V67, V127, and V152

Group	T-cell clone	Proliferative response ^a to:			
		V36	V67	V127	V152
1	Mem clone 1	66.7 ^b	6.4	169.6	74.7
2	F1-40	99.9	52.2	6.1	43.1
	F1-33	69.8	40.6	5.2	65.3
	F1-70	79.9	82.9	10.7	44.7
3a	F1-51	73.9	66.6	9.2	53.3
	F1-52	72.6	66.3	11.5	40.5
	F1-5	87.3	50.6	12.8	51.1
3b	F1-48	84.4	63.4	32.9	67.2
	F1-55	98.6	58.3	24.3	72.1
	F1-36	80.5	69.4	32.7	76.4
4	HA-1	74.4	68.2	36.2	6.6
	HA-3	75.2	64.4	55.7	10.5
	HA-7	71.8	85.2	37.5	26.5
	HA-11	79.6	82.7	31.2	21.2

^a Cultures contained 10 HAU of virus.

^b Data represent mean cpm of triplicate cultures expressed as a percentage of the value obtained for cultures containing Mem 71. Values of <15% are printed in boldface, and those >15% but <40% are italicized.

V67 greatly diminished the ability of the group 1 clone to mount a proliferative response but had little or no effect on any of the other T-cell clones. The change in V127 affected the proliferation of clones in the other groups, the reduction being more pronounced with groups 2 and 3a (in which the proliferation was <15% of that with Mem 71 virus) than with groups 3b and 4 (in which the response averaged 35% of that with Mem 71). In addition to diminished reactivity with V127, each of the group 4 clones also showed a reduced response to V152. The mutant virus V36, although antigenically related to the other three mutants, was able to stimulate all of the T-cell clones.

Location of amino acids critical to the integrity of the epitopes recognized by T cells. The location and nature of the amino acid substitutions in escape mutants V67, V127, and V152 were determined by sequencing their HA genes by using the dideoxy method (24). It was found that V67 differs from the parent virus Mem 71 only at residue 60 in HA₁, which changes from Asp to Val. Mutants V127 and V152 both have changes at residue 63. In V127, this is an Asp → Asn substitution, creating a potential glycosylation site at Asn-63-X-Thr-65, as is found in the later naturally occurring H3 strains and in Eng 69. In V152, the change is Asp-63 → Tyr. Both residues 60 and 63 are located in the region of HA known as site E (7) and are shown diagrammatically in Fig. 3.

DISCUSSION

The T-cell clones raised for this study came from three independent sources, namely from the spleens of a BALB/c mouse and a (BALB/c × C57BL/10)F₁ mouse which had been immunized intraperitoneally with infectious Mem 71

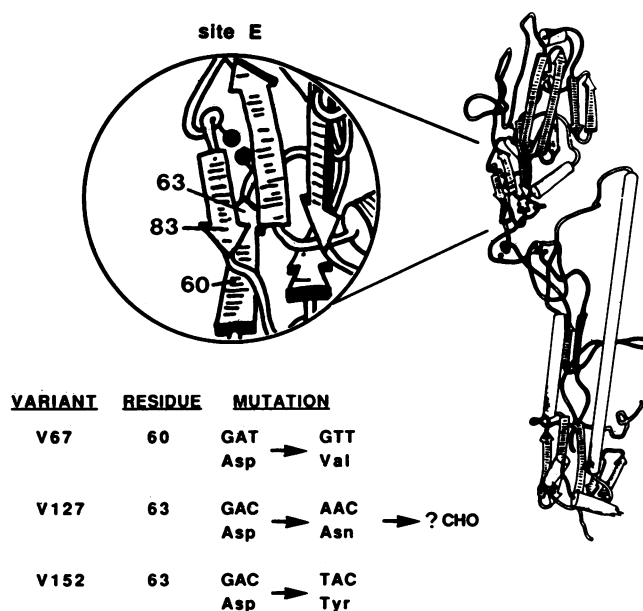


FIG. 3. Diagram of a monomer of the H3 HA drawn from the X-ray crystallographic data of Wilson et al. (37) with the antigenic region known as site E enlarged. The location of the amino acid substitutions in MAb-selected mutants that failed to stimulate one or more of the T-cell clones is indicated, as is residue 83 which is implicated in the epitope of the group 1 clone by its pattern of reactivity with natural field strains. The mutation in V127 leads to the creation of a potential site of carbohydrate (CHO) side chain attachment.

	55	60	65	70	75	80	85
DUCK 63	-	-	-	-	-	-	-
ENG 69	-	-	-	-	-	-	-
MEM 71	P	H	R	I	L	D	G
MEM 72	-	-	-	-	-	-	-
PC 73	-	-	-	-	-	-	-
VIC 75	-	-	-	-	-	-	-
TEX 77	-	-	-	-	-	-	-
BANG 79	-	-	-	-	-	-	-

FIG. 4. The amino acid sequences of the site E region of HA of various natural field strains of the H3 subtype. The amino acid sequence of Mem 71 is shown, and for the other strains, a dash is used to indicate the same residue as in Mem 71. The single-letter code for amino acids is used. Sequence data are as shown in the review by Ward (33).

(H3) virus and from pooled lymph nodes from seven BALB/c mice immunized in the footpads with purified HA. Despite the different sources, all 14 of the clones, found to be specific for the HA of influenza A virus of the H3 subtype, recognized epitopes whose integrity was lost in escape mutants containing an amino acid substitution in residue 60 or 63 of HA₁. Therefore, this region of the HA molecule, which lies within what is known as site E (7), clearly represents a major antigenic site for H-2^d class II MHC-restricted T cells. Within this site, at least four different epitopes recognized by T cells were defined by the distinct patterns of specificity of the clones for the panel of naturally occurring virus strains tested.

Additional information about the nature of each epitope can be derived by comparing the HA amino acid sequences of those viruses that stimulate a given T-cell clone with those that do not. The HA sequences of all of the viruses except PNG 75 and Phil 82 have been reported (33) and are shown, for the site E region, in Fig. 4. Mem clone 1 (group 1) is unique in that its reactivity is abolished by the substitution Asp → Val at residue 60 in V67 but is not affected by the substitutions at residue 63 in V127 and V152. Its failure to respond to field strains isolated from 1975 onward also implies sensitivity to one of the amino acid changes in HA₁ that occurred after 1973, namely in residue 83, 126, 189, or 278. Although none of these are near residue 60 on the linear sequence, examination of a three-dimensional model of HA (Fig. 3; 37) shows that amino acids 60 and 83 lie adjacent to one another on antiparallel chains.

The amino acid substitution in V127 affects the reactivity of all of the other clones to some extent. The response of group 2 T-cell clones is the most affected, and this is paralleled by a total lack of reactivity of these clones with all naturally occurring strains that have acquired the same change (Asp-63 → Asn) as a result of antigenic drift. Group 3a clones also show a marked decrease in response both to V127 and to the field strains with the equivalent substitution at residue 63 but, in addition, respond very poorly to Duck 63. This tends to implicate residue 62 (Arg in Duck 63, Ile in Mem 71) but is not inconsistent with possible involvement of residues 78 or 81 or both in the epitope as well.

Group 3b and group 4 T-cell clones are also affected by the change in V127, but their proliferation is reduced rather than abrogated. Likewise, they are more tolerant of this substitution in field strains. The epitopes recognized by these two groups of clones may also include residue 62, as the response to Duck 63 is somewhat diminished, but they differ in other respects. Tex 77 and Bang 79, which have changes at both residues 62 and 63, fail to stimulate group 3b clones but are still recognized by the group 4 clones, which cross-react with all the field strains of the H3 subtype. Moreover, group 4 clones are characterized by a reduced response not only to V127 (Asp-63 → Asn) but also to V152 (Asp-63 → Tyr). This

suggests that residue 63 is actually within the epitope for group 4 clones. While this may also be the case for the epitopes recognized by clones of groups 2, 3a, and 3b, it is less likely because the change in V152 (Asp-63 → Tyr) is tolerated by these clones. An alternative explanation is that an oligosaccharide side chain may be attached at the new potential glycosylation site generated by the Asp-63 → Asn change in V127 and may affect various regions close to residue 63 even though the epitopes may not involve residue 63 itself. While the glycosylation pattern of the HA molecule is not known for V127 nor for all of the relevant field strains of the H3 subtype, Eng 69 and Vic 75 at least are known to be glycosylated at residue 63 (28). Carbohydrate at this position has been shown to prevent recognition of certain epitopes by antibody (28), presumably by steric hindrance. If processing of antigen by APC fails to remove carbohydrate, then T-cell epitopes on fragmented or denatured HA may similarly be masked. Alternatively, glycosylation may prevent the appropriate proteolytic cleavage necessary for the correct presentation of particular epitopes or the required interaction with the class II MHC (Ia) molecule.

It is striking that all of the epitopes described here are situated in the same region of the HA molecule and are probably even overlapping. A similar relationship between myoglobin epitopes for T cells has been reported by Cease et al. (6), who suggest that the immunodominance of a T-cell site depends not on the dominance of clones recognizing a single epitope but on the focusing of a polyclonal response, made up of multiple T-cell specificities arising from multiple views of a single processed peptide, presented at a single Ia-binding site. The fact that all of the site E-specific clones described here are *I-A^d* restricted, as well as being affected by changes in residues within the same region of the HA molecule, is consistent with the hypothesis that their different epitopes may utilize a common agreptope for interaction with the *I-A^d* molecule. There are data with other influenza HA-specific T-cell clones which could also be interpreted in this way. Hurwitz et al. (16) and Hackett et al. (15) described different fine specificities among *I-E^d*-restricted clones reacting with a synthetic peptide corresponding to residues 109 to 119 of the HA₁ of the PR8 strain of subtype H1. Also, Thomas et al. (31) in a recent report have described five different *I-A^k*-restricted clones from CBA mice which recognize the HA of X-31 (H3) virus but not a MAb-selected mutant with a change at residue 135. Four of the five clones responded to a peptide encompassing this residue, yet each displayed a unique fine specificity as assessed by reactivity with field strains.

Attempts to define the limits of the site E epitopes recognized by our T-cell clones by using synthetic peptides have been unsuccessful. None of the clones reacted with peptides corresponding to HA₁ residues 48 to 68 or 53 to 68. One possibility is that these peptides are not long enough at the C-terminal end and require residues beyond 68 either for the epitope or for the agreptope. Alternatively, the T-cell clones may not recognize the site E region itself, but changes there may affect the processing or presentation of other nearby or more distant regions of the molecule. A third possibility is that the T cells must recognize conformational features of the native HA that are not present in the synthetic peptide. Mills et al. (22) have provided evidence for this with certain of their HA-specific T-cell clones and on this basis have postulated alternative routes of antigen processing that do not involve intracellular cleavage into shorter peptides. The group 1 T-cell clone described in this report may recognize such a conformational (or assembled)

epitope involving residues 60 and 83, which are very close together in the intact HA molecule though far apart in the linear sequence.

The recognition of the site E (or equivalent) region by murine T cells has been reported only for influenza viruses of the H3 subtype, in which it also forms a major site of interaction with antibody. As well as the BALB/c (*H-2^d*) clones described in this report, CBA (*H-2^k*) clones that recognize this region have recently been described. Six clones isolated by Mills et al. (23) respond to a synthetic peptide (residues 48 to 68 of X-31), and judging from their patterns of reactivity with naturally occurring field strains, require residue Ile-62 (site E) and possibly also residues Asn-53 and Asn-54 (site C) for interaction with HA. These six CBA clones recognize an epitope that clearly differs from any of the epitopes described in this paper because they fail to recognize Duck 63 yet do recognize Eng 69, PC 73, and Vic 75 and thus do not apparently involve residue 63, 78, 81, or 83. The restriction element utilized by CBA mice (*H-2^k*) may present a somewhat different spectrum of epitopes to the set presented by *I-A^d*. Thomas et al. (30) have recently devised a negative selection (suicide) procedure to obtain murine T-cell clones specific for the variable regions of HA, which they have applied to T-cell lines from BALB/c mice primed with X-31 (H3) virus to eliminate those T cells that could also respond to the closely related Eng 69 strain. Clones were then isolated that had the desired reactivity with X-31 but not Eng 69 and that, by inference, recognized one of the three amino acids in HA₁ (Asn-31, Asn-63, or Asp-81) that differ between these two viruses.

In this study, we have shown that site E on the influenza H3 HA molecule is a major recognition site for *H-2^d* T_H cells and that this site comprises at least four probably overlapping epitopes which can be distinguished by the reactivity patterns of different T-cell clones with panels of naturally occurring field strains and further characterized by using MAb-selected laboratory mutants. Some of these clones are relatively specific for the parent strain of virus, whereas others are broadly cross-reactive with strains that have arisen by antigenic drift over many years.

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