Identification of Eight Determinants in the Hemagglutinin Molecule of Influenza Virus A/PR/8/34 (H1N1) Which Are Recognized by Class II-Restricted T Cells from BALB/c Mice

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Eight nonoverlapping regions of the hemagglutinin (HA) molecule of influenza virus A/PR/8/34 (PR8), which serve as recognition sites for class II-restricted T cells (T_H) from BALB/c mice, have been identified in the form of 10- to 15-amino-acid-long synthetic peptides. These T_H determinants are located between residues 110 to 313 of the HA₁ polypeptide. From a total of 36 HA-specific T_H clones and limiting-dilution cultures of independent clonal origins, 33 (90%) responded to stimulation with one of these peptides. The residual three T_H clones appeared to recognize a single additional determinant on the HA₁ polypeptide which could not be isolated, however, in the form of a stimulatory peptide. None of the motifs that have been proposed to typify T_H determinants were displayed by more than half of these recognition sites. Most unexpected was the finding that none of the T_H determinants was located in the ectodomain of the HA₂ polypeptide that makes up roughly one-third of the HA molecule. Possible reasons for the preferential recognition of HA₁ as opposed to HA₂ by T_H are discussed.

Current evidence indicates that T_H cells recognize complexes formed between short segments of protein antigens and class II molecules of the major histocompatibility complex (4, 14, 15, 51, 55, 60). To expose these segments for effective interaction with class II molecules (53), most protein antigens must undergo certain structural alterations that normally take place in the antigen-presenting cell (APC) and are globally referred to as antigen processing (2, 58). The fact that T_H recognition sites can usually be isolated from intact proteins in the form of short peptides has made possible detailed specificity analyses of T_H responses to many proteins. These studies have begun to reveal some of the principal factors that influence the selection of specific protein regions for recognition by T_H . These include (i) the presence of structural properties (motifs) in the antigen that are thought to mediate binding of the given polypeptide regions to class II molecules (40, 47, 52, 54); (ii) processing mechanisms that may result in the destruction or preferential exposure (1, 8, 28, 61) of distinct protein regions, in part perhaps depending on the form in which antigen is delivered or on the main type of APC involved in processing (19, 20, 26, 39, 42, 64); and (iii) the major histocompatibility complex haplotype of the responding organism, which selects for the best binding determinant regions or affects the T_H repertoire available for response (7, 15, 35, 41, 48). At present, none of these mechanisms is understood in sufficient depth to predict with satisfactory accuracy the specificity of a T_H response to a given protein.

In our previous analyses of the T_H response of BALB/c mice to the hemagglutinin (HA) molecule of influenza virus A/PR/8/34 (PR8), we had identified three regions of the protein (designated sites 1, 2, and 3) that were recognized by a panel of eight T_H clones (32). These T_H sites were located in the HA₁ polypeptide and displayed distinct processing

requirements and rates of catabolism (24, 25). To obtain further insight into factors that influence determinant selection in this response, we wished to investigate whether these three determinants were the sole regions of HA to which BALB/c mice could respond. Here we describe the analysis of 28 additional HA-specific T_H clones and limiting-dilution cultures, each of independent clonal origin and derived from mice after immunization with virus or viral glycoproteins. These T_H clones led to the identification of five additional T_H sites of the HA. Their locations and structural properties are discussed.

MATERIALS AND METHODS

Mice. Female BALB/c mice (Harlan Sprague Dawley, Indianapolis, Ind.; Jackson Laboratory, Bar Harbor, Maine) were used at the age of 2 months or older.

Viruses and viral antigens. The influenza viruses were used in the form of allantoic fluid from virus-infected embryonated hen eggs. Virus titers were determined by agglutination of chicken erythrocytes and are expressed in hemagglutinating units (HAU). Soluble HA trimers (BHA) were prepared and purified as described by Brand and Skehel (11), and protein concentration was determined by the Bio-Rad protein assay. Purified HA₁ was kindly provided by C. Hackett, Wistar Institute.

Peptides. All peptides were produced in the Peptide Synthesis Facility of the Wistar Institute, using automated solid-phase methodology. The peptides were tested for amino acid composition and were purified by reverse-phase chromatography on a C18 column (Waters, Milford, Mass.). The peptides were dissolved at a concentration of 10^{-3} M in phosphate-buffered saline containing 1 mg of bovine serum albumin per ml, filter sterilized (0.2- μ m-pore-size filter), and stored frozen. In the case of S6, which contains two cysteines (Table 1), 2-mercaptoethanol (0.5×10^{-3} M) was added to the solvent to prevent oxidation.

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Generation of T_H clones. Various immunization protocols and culture procedures were used during this study to generate virus-specific T_H clones. The derivations of the standard clones used most extensively to identify the respective sites were as follows. The Vir series (V1.2; anti-S1) was generated as described previously (29) from the spleen of a mouse taken 12 days after intraperitoneal injection of 100 HAU of PR8 in phosphate-buffered saline; the T2 (T2.5-5; anti-S2) and TL35H (TL35H-6; anti-S8) series were derived from draining lymph nodes removed 8 to 10 days after subcutaneous (s.c.) injection of 100 and 2,000 HAU, respectively, of PR8 in complete Freund adjuvant (CFA) (49); series 7 (7.1-5; anti-S4) and 8 (8.1-6R8; anti-S3) were derived from the draining lymph nodes of two mice that had been injected s.c. with PR8 in CFA following recovery from a pulmonary infection with PR8; the MT2B (MT2B-11.1; anti-S7) series was derived from the lymph node of a mouse injected s.c. with 2,000 HAU of PR8 in CFA and boosted 4 weeks later by s.c. injection of 1,000 HAU of PR8 in incomplete Freund adjuvant into the same site. In all of these cases, initial mass cultures were set up essentially as described previously (29) and cloned by limiting dilution. The LD1 (LD1-4.7; anti-S5) and LD2 (LD2-14.6; anti-S6) series were derived from draining lymph nodes obtained 7 to 10 days after s.c. injection of 2,000 HAU of PR8 in CFA; in these cases, however, the lymph node cells were initially seeded at limiting dilutions into microcultures (500 to 2,500 cells per culture in 96-well round-bottom plates) containing 2×10^6 APC-V per ml (see below).

Maintenance of T_H clones. T_H cells (2×10^5 /ml) were stimulated by incubation (37°C; humidified air-7% CO₂) with irradiated (2,200 rads) virus-pulsed spleen cells (APC-V; 2×10^6 /ml; see below) in Iscove modified Dulbecco medium (GIBCO, Grand Island, N.Y.) supplemented with transferrin (0.005 mg/ml; Sigma Chemical Co., St. Louis, Mo.), 2-mercaptoethanol (5×10^{-5} M), gentamicin (0.05 mg/ml), bovine serum albumin (0.01%; Boehringer Mannheim Biochemicals, Indianapolis, Ind.), soybean lipid (0.05%; Boehringer Mannheim), and fetal calf serum (2%; Hyclone, Logan, Utah) (Isc-CM-2%). After 3 to 5 days, the cultures were supplemented with 1/3 volume of Isc-CM-5% which additionally contained 1% CAS (supernatant obtained from rat spleen cells cultured for 24 h in the presence of 5 µg of concanavalin A per ml) and 1% each interleukin-2- and interleukin-4-containing culture fluids from the corresponding X63Ag8-653 transfectant cell lines (33) (Isc-R). After further incubation for 1 to 3 days (depending on cell growth), viable cells were separated from dead cells by centrifugation onto a cushion of Ficoll-Hypaque and cultured at $\sim 10^6$ cells per ml in Isc-R. Depending on cell growth, one half of the medium was replaced (or culture expanded) at 3- to 5-day intervals. T_H were usually restimulated as described above with APC-V at 3- to 6-week intervals. If they were kept for longer than 3 weeks without restimulation, the cultures were maintained at 32°C in Isc-R with 50% medium replacements at 7- to 10-day intervals. T_H were used for assays at or past 10 days after restimulation with APC-V.

APC-V were prepared by incubating a suspension of irradiated (2,200 rads) spleen cells (20×10^6 cells per ml in Iscove medium without serum) with influenza virus-containing allantoic fluid (~ 1 HAU per 10^6 spleen cells). After 1 h at room temperature, the cells were pelleted, the supernatant was discarded, and the virus-pulsed cells were resuspended at the desired concentration in the final culture medium.

T-cell assays. Analysis of restriction specificity was performed by using the I-A^d and I-E^d transfected L-cell lines

(30) as APC and monitoring T-cell activation by measuring interleukin-3 secretion as described previously (31a). Proliferation assays were done by measuring [³H]thymidine incorporation, using irradiated (2,200 rads) spleen cells from naive BALB/c mice as APC. Stimulation indices were computed by dividing counts per minute observed in the presence of specific antigen by counts per minute observed in control cultures (medium alone).

Statistical analyses. Chi-square analysis was used to compare the amino acid compositions and secondary structures of T_H determinants with those of non-T_H determinant regions of the HA₁ polypeptide. The AMPHI program (40) was used to determine amphipathic indices for helical conformations with periodicity around 100° (A1) and around 120° (A2). The data were plotted by taking the sum of the indices from three consecutive blocks, using in each block the higher index value. The resulting amphipathic score (AS) was plotted along with the average of column 7 of the AMPHI printout, which indicates whether a given block can (1) or cannot (0) form an amphipathic helix with periodicity between 80° and 135°.

RESULTS

Immunization of BALB/c mice with influenza virus PR8 induces a T_H response to at least 8 nonoverlapping regions on the HA₁ polypeptide. We had found previously that eight HA-specific T_H clones, generated from six PR8-primed BALB/c mice by long-term culture or fusion with BW5147, were specific for one of three regions of the HA₁ polypeptide (32). The corresponding T_H determinants were subsequently isolated in the form of synthetic peptides and were designated T_H sites 1, 2, and 3 (24) (referred to here as S1, S2, and S3).

To investigate whether these three sites were the only determinants on HA to which T_H of BALB/c mice could respond, we proceeded as follows. T-cell mass cultures and limiting-dilution cultures were generated from BALB/c mice immunized with intact PR8 virus or viral glycoproteins. Established cultures were screened for the presence of HA-specific T_H by testing their proliferative responses to stimulation with BHA (HA released by action of bromelain from viral lipid envelope; BHA lacks the C-terminal transmembrane and intracytoplasmic regions of HA₂). Cultures that contained HA-specific T_H cells were further tested for the ability to proliferate in response to the peptides S1, S2, and S3 and the subsequently isolated T_H determinants as they became available. Cultures that failed to respond to any of the available T_H determinants were cloned and further tested for response to a panel of 49 PR8 mutant viruses. Each of these mutant viruses differs from PR8 by one to two unique amino acid substitutions in the HA₁ polypeptide. The failure of T_H clones to respond to one or a group of PR8 mutants provided in most cases (see below) initial evidence for the approximate location of the HA region recognized by the clones. The final characterization of each T_H recognition site was made by synthesizing 10- to 15-amino-acid-long peptides corresponding to the suspected HA regions and demonstrating that these peptides stimulated the given T_H clone(s) upon presentation by syngeneic splenic APC.

Eight distinct T_H specificities, numbered in chronological order of isolation, could be defined by means of synthetic peptides (Table 1). Each of these peptides corresponds to a unique segment of the HA₁ polypeptide. Of 36 clonally independent HA-specific T_H isolates from virus- or glycoprotein-immunized BALB/c mice, 33 responded to one of

TABLE 1. Determinants recognized by HA-specific class II-restricted T cells isolated from PR8-immunized BALB/c mice^a

Site	HA ₁ region ^b	Sequence ^c	Restriction element ^d	No. of independent T _H isolates ^e
S1	110-120	SFERFEIFPKE *	E	7
S2	126-138	HNTNGVTAACSHE *	A	11
S3	302-313	CPKYVRS AKLRM	E	5
S4	159-170	KLKNSYVNKKGK * * * * *	E	4
S5	195-209	NAYVSVTSNYYRRF *	A	2
S6	269-280	ASMHECNTKCQT	A	2
S7	212-224	EIAERP KVRDQAG **	A	1
S8	174-185	VLWGIHHPNSK * * *	E	1
Sx	1-325		A	3

^a Designations and sequences of the synthetic peptides that are recognized by HA-specific limiting-dilution cultures and T_H clones from BALB/c mice are shown. The minimum determinant region has been defined only in the case of S1 and comprises, depending on T_H clone, amino acids 110 to 119 or 111 to 119 (31a).

^b Numbers indicate N- and C-terminal amino acid positions in the H1 subtype HA.

^c Residues marked with asterisks were found to be altered in viral escape mutants selected in the presence of monoclonal antibodies.

^d The class II isotype used in the presentation and recognition of the peptides was determined by means of class II-transfected L cells as described in Materials and Methods.

^e Number of distinct donor mice from which T_H responding to the indicated synthetic peptides were isolated.

these eight peptides (T_H clones of the same specificity were considered to be clonally independent only if isolated from a different donor mouse).

Three of the HA-specific clones (Table 1, specificity group Sx) failed to respond to any of these peptides. However, although these three clones (LD2-23, LDA2-F8, and 5.1-5) did not respond to peptide S6, they exhibited specificity patterns very similar to those displayed by the S6-specific T_H clones. Thus, the S6- and Sx-specific clones responded well to purified HA₁ polypeptide but not to reduced and alkylated HA₁ (Table 2), suggesting the presence of a cysteine within the Sx determinants, as is the case with S6 (Table 1). None of these clones responded to peptides that overlapped with S6 in the N- or C-terminal region (Table 2). Furthermore, the S6- and Sx-specific clones responded to all antibody-selected PR8 mutants (data not shown), were re-

stricted to I-A (Table 1), and displayed indistinguishable response patterns against a panel of 26 epidemic virus strains of the H1 subtype, which each display multiple amino acid differences from PR8 (Fig. 1). In the case of virus strain 5 (WSA), one of the S6-specific T_H clones (LD2-14.6) and two of the Sx-specific clones gave a positive response (stimulation index of >3), while one S6-specific and one Sx-specific clone failed to respond. Taken together, these findings are consistent with the idea that the three Sx-specific T_H clones are directed to the same general region of HA as are the two S6-responsive T_H clones but that the corresponding determinants may depend on intact disulfide bonds, of which two are present in this region of the protein.

The various peptides differed considerably in stimulatory potency and did not substitute equally well for the corresponding determinants generated from intact protein by intracellular processing mechanisms (Fig. 2). Nevertheless, with the exception of S6, these peptides induced 50% maximum stimulation at concentrations of 0.1 to 100 nM, which represent average to high stimulatory potencies compared with those of peptides reported in other experimental systems. Regarding the comparison between peptides and BHA, note that BHA is a homotrimer and contains 3 mol of any given T_H determinant per 1 mol of protein.

Structural characteristics of the T_H sites. Several motifs that may underlie the affinity of T_H determinants for class II have been proposed. Margalit et al. (40) proposed that the ability for formation of a stable amphipathic helix is a general structural property related to T_H determinant function. However, except for S1 and S3, the T_H determinants identified in this study cluster into a region of HA₁ with low AS (see legend to Fig. 3).

If a block length of 7 was used to assess the AS of isolated peptides, only S3 and S5 contained three consecutive blocks compatible with amphipathic helical structure and exhibited AS above the threshold value of 8 (Table 3), which are the two requirements given by Margalit et al. (40) for formation of a stable amphipathic helix. Both of these peptides exhibited high stimulatory potencies. The motif proposed by Rothbard and Taylor (47) was present in three of the determinants. Finally, the I-A^d and I-E^d restriction motifs defined by Sette and collaborators (52, 54) were displayed by half of the respective T_H determinants and were present in each case in the determinants with the highest stimulatory potencies.

The structural characteristics of the T_H sites were further assessed by comparing them with those parts of the HA₁ polypeptide (227 amino acids) that do not contain known T_H determinants (Table 4). Using secondary-structure informa-

TABLE 2. Relationship between S6- and Sx-specific T_H clones^a

Antigen	Stimulation index displayed by T _H clone:				
	LD2-14.6 (anti-S6)	LD1-11 (anti-S6)	LD2-23 (anti-Sx)	LDA2-F8 (anti-Sx)	5.1-5 (anti-Sx)
PR8	77	7	32	76	227
HA ₁	157	12	24	80	125
HA ₁ , reduced and alkylated	1.2	ND ^b	1.1	1.6	1.7
GIITSNASMHEC	0.8	1.3	ND	0.5	0.4
ASMHECNTKCQT (=S6)	26	3.6	1.6	1.3	1.1
CNTKCQTPLGAI	1.3	0.8	ND	1.0	0.6

^a T_H clones were tested in standard proliferation assays. Average [³H]thymidine incorporation of triplicate samples is expressed as stimulation index, determined as described in Materials and Methods. Stimulation index values of ≥3 are considered significant. All antigens were used at a concentration that induced (near) maximum proliferation with these or other suitable control T_H clones. The peptides were used at 10 μM.

^b ND, Not determined.

FIG. 1. Proliferative responses of S6- and Sx-specific T_H clones to epidemic virus strains. T_H clones were tested in the standard proliferation assay (see Table 2, footnote *a*) for their responses to 26 epidemic virus strains of the H1 subtype. The responses to 11 of these viruses, originally isolated between 1931 and 1943, are shown: SW/31 (1); four virus strains derived from WS/33, i.e., WSN (2), NWS (3), WSE (4), and WSA (5); PR/8/34 (6); BH/35 (7); MEL/35 (8); Hickcox/40 (9); BEL/42 (10); and Weiss/43 (11). All T_H clones failed to respond (data not shown) to the following viruses: CAM/46, FM/1/47, Roma/49, FW/1/50, England/1/51, FLW/1/52, Malaya/302/54, Denver/57, Mayo Clinic/103/74, New Jersey/10/76, USSR/77, Lackland/78, Brazil/78, England/333/80, and India/6263/80. Each virus was used at a 1:1,000 dilution of virus-containing allantoic fluid. All viruses stimulated appropriate control T_H (data not shown). Stimulation indices were computed as described in Materials and Methods. Stimulation indices of ≥ 3 are considered significant.

tion obtained from the X-ray crystallographic analysis of the H3 subtype HA (63; kindly provided by Ian Wilson, Scripps Clinic and Research Foundation), no significant preference for particular secondary structures was evident. However, T_H sites differed significantly from non-T_H regions in that they had an increased content in positively charged amino acids and, among the group of nonpolar hydrophobic amino acids, had a preference for alanine, phenylalanine, methionine, and valine rather than for leucine and isoleucine.

Location of T_H sites on the 3D model of the HA molecule. The three-dimensional (3D) structure of the HA molecule of the H3 subtype (strain Aichi/68) has been determined by Wilson et al. (63). Two domains can be distinguished on the HA monomer: (i) a fibrous stem structure that anchors the

molecule into the lipid membrane of the virion and is formed by HA₂ and short C-terminal (approximately amino acid positions 280 to 325) and N-terminal (approximately amino acid positions 1 to 40) regions of HA₁ and (ii) a globular distal domain that is formed by the main portion of HA₁ (approximately amino acid positions 55 to 265). Two antiparallel strands of HA₁ (hinge region) connect the two domains. Although the H1 subtype HA of PR8 differs in sequence from the H3 subtype by 65 and 47% in the HA₁ and HA₂ polypeptides, respectively, the two HA subtypes share many key structural features and appear to possess very similar 3D structures (17). Following alignment (17) of the PR8 sequence with the H3 subtype sequence, the T_H determinants were projected onto the 3D structure. Six of the

FIG. 2. Stimulation potencies of BHA and peptides. T_H clones were tested in parallel for proliferation to a range of concentrations of BHA and the specific peptides. Because BHA is a homotrimer, 1 mol of BHA would correspond to 3 mol of a given T_H determinant. A representative experiment is shown for each T_H clone. Each data point is the average of triplicate cultures.

FIG. 3. Relationship between amphipathicity and location of T_H determinants. Amphipathicity of HA_1 and HA_2 was determined by using the AMPHI program. The AS values were computed from three consecutive 11-amino-acid blocks (upper line). The data in column 7 of the AMPHI output, which indicates with 1 the blocks with maximum amphipathicity in 3_{10} or alpha-helical conformation and with 0 all other blocks, were averaged (lower line). AS values of >4 over at least three consecutive blocks in 3_{10} or alpha-helical conformation (lower line = 1) indicate, according to Margalit et al. (40), propensity for stable amphipathic helical conformation. Solid blocks indicate locations of T_H determinants.

TABLE 4. Structural characteristics of T_H sites^a

Characteristic	No. (%) of residues of HA_1		
	In T_H sites	In other regions	P
Alpha helix	3 (3)	19 (8)	NS
Beta sheet and bends	57 (58)	115 (51)	NS
Random coil	38 (38)	93 (41)	NS
His, Lys, Arg	23 (23)	18 (8)	<0.001
Leu, Ile	6 (6)	42 (19)	<0.005
Ala, Phe, Met, Val	21 (21)	25 (11)	<0.05

^a The probability of the observed composition of the presently known T_H sites (98 amino acids) versus other sites of the HA_1 polypeptide (227 amino acids) was assessed by chi-square analysis. P indicates the likelihood that this observation is made by chance. NS, Not significant. The content in secondary structure is based on X-ray crystallographic data made available by Ian Wilson, Scripps Clinic and Research Foundation.

sites (S1, S2, S4, S5, S7, and S8) are located in the globular domain, S6 is located in the hinge region, and S3 is located in the distal portion of the stem (Fig. 4).

The main B-cell recognition sites are also located in the globular domain of HA (17, 62). In the case of PR8, these B-cell epitopes have been identified by determination of amino acid substitutions that permit escape of PR8 mutants from neutralization by individual monoclonal anti-HA antibodies. The currently available panel of PR8 escape mutant viruses consists of 49 viruses, each differing from PR8 by a unique amino acid substitution at one of 38 distinct positions of HA_1 . Accepting that residues with escape mutations delineate parts of B-cell epitopes, the number and frequency of these residues within T_H sites would provide a measure for the overlap between B-cell and T_H determinants in this protein. Two T_H determinants (S3 and S6/Sx) contain no residues with escape mutations, while the others contain one (S1, S2, S5), two (S7), three (S8), and seven (S4) such residues (asterisks in Table 1).

Within the six T_H sites present in the globular region of HA (~210 amino acids of HA_1), each of which contains at least one residue with an escape mutation, the average frequency of escape mutations is 19.7% (15 of 76 amino

TABLE 3. Presence of motifs in T_H sites

Peptide ^a	Stimulatory potency (nM) ^b	AS ^c	Presence of motif ^d		
			Rothbard and Taylor	Sette et al.	
				A ^d	E ^{d,e}
I-E restricted					
S4 (KLKNSYVNKKGK)	0.21	12.7	—	—	+
S3 (CPKYVRS AKLRM)	0.24		+	—	+
S8 (VLWGIHPPNSK)	24		—	—	+/—
S1 (SFERFEIFPKE)	92		+	—	—
I-A restricted					
S5 (NAYVSVVTSNYNRRF)	0.10	16.3	—	+	—
S2 (HNTNGVTAACSHE)	2		—	+	—
S7 (EIAERPKVRDQAG)	10		+	—	+
S6 (ASMHECNTKCQT)	~20,000		—	—	+/—

^a Grouped according to restriction element and stimulatory potency.

^b Concentration at which the peptide induces 50% maximum proliferation upon presentation by irradiated (2,200 rads) syngeneic splenic APC. Values are means of three to five independent determinations using the standard peptide/ T_H combinations S1/V1.2, S2/T2.5-5, S3/8.1-8R8, S4/7.1-5, S5/LD1-4.7, S6/LD2-14.6, S7/MT2B-11.1, and S8/TL35H-6.

^c Computed by using the AMPHI program (40). Each value is the sum of the amphipathic indices (block length of 7) over the entire peptide region. Values are for peptides that contained at least three consecutive blocks with amphipathicity in 3_{10} or alpha-helical conformation.

^d Evaluated as described by the indicated authors (47, 52, 54).

^e +, Presence of a motif according to the three basics rule (52); and +/-, presence of two basic amino acids separated by not more than five intervening nonbasic amino acids.

FIG. 4. Schematic drawing of the BHA structure of H3 subtype HA according to Wilson et al. (63). HA₁ polypeptide is shown by continuous lines, and BHA₂ is shown by broken lines. The regions of HA₁ that contain a T_H determinant are indicated.

acids). If there were a preferential overlap between T_H and B-cell recognition sites, one would expect the frequencies of escape mutations to be similar in the two sites. The frequency of the present set of escape mutations within B-cell determinants cannot be precisely estimated since the number of residues that are directly involved in recognition by antibodies is not known. However, the latter is likely to be much higher than the average frequency of escape mutations per the entire globular domain, i.e., 18.1% (38 of 210 amino acids), because only a fraction of the ~210 amino acids of the globular domain of HA are accessible for direct interaction with antibody-combining sites. Since the frequency of escape mutations in T_H recognition sites (19.7%) is similar to their average frequency over the entire globular domain (18.1%) and probably substantially smaller than their frequency per B-cell recognition site, it appears that the observed overlap between T_H sites and B-cell sites is coincidental rather than causal.

FIG. 5. Synthetic peptides that are recognized by T_H cells from *H-2^d* mice induced by immunization with influenza virus or HA of the H1, H2, or H3 subtype (■). *, Amino acid substitution that abolished recognition of the respective HA by T_H; □, T_H were induced by immunization with the given synthetic peptide, but one of the resulting T_H clones reacted also with naturally processed HA. The location of each T_H recognition site is indicated in relation to a diagram of HA₁ that also shows the locations of the various disulfide bonds. Positions are given according to the H3 subtype numbering system. All studies except that described in reference 3 are based on analysis of T_H clones. The study described in reference 3 is based on restimulation of lymph node T-cell mass cultures from virus-primed mice with synthetic peptides.

DISCUSSION

This analysis shows that the HA-specific T_H response of BALB/c mice is directed to at least eight distinct nonoverlapping regions of the 325-amino-acid-long HA₁ polypeptide. Although 3 of the 36 T_H clones failed to respond to any of the peptides that represent these sites, it is likely that these residual T_H clones are directed to the same general region as that identified by the S6 peptide because they are concordant with the two S6-responsive T_H clones in their reactions against many different forms of the HA (Table 2 and Fig. 1). Since the determinant recognized by both S6- and Sx-specific T_H clones was destroyed upon alkylation of reduced HA₁, it is possible that the intact and fully active T_H site, as generated from BHA by normal processing, additionally requires one or both of the disulfide bonds present in this region of the HA and may further require, in the case of Sx-specific T_H, other amino acid residues provided by the disulfide-linked polypeptide segments. It is noteworthy that Cys-274 of the S6 site is linked to Cys-42 in the native HA. In the HA of the H3 subtype, the latter region has been shown to contain several determinants that are recognized by T_H cells from *H-2^d* mice (3, 5, 12) (Fig. 5). Interestingly, Brown et al. (12) were not successful in their attempts to isolate the corresponding determinant in the form of a synthetic peptide and suggested that T_H may recognize a "conformational" determinant in this region. Thus, this general region of HA may be a major focus for the T_H response of *H-2^d* mice, but many of the T_H epitopes recognized in this region may depend on intact disulfide bonds.

Taken together, the data indicate that the eight peptides identify (at least partially in the case of S6) all sites recognized by the 36 T_H clones in the panel. Furthermore, because we have used many different protocols in the generation of this T_H panel and because T_H clones of all except two specificities (S7 and S8) were repetitively isolated from different donor mice, it is likely that the analysis provides a nearly exhaustive description of the entire HA-specific T_H response of BALB/c mice. It is noteworthy that a total of seven nonoverlapping T_H recognition sites in HA₁

have been identified in the combined studies of three different groups (3, 5, 12) who explored the response of *H-2^d* mice to the HA of the H3 subtype (Fig. 5).

None of the structural motifs that have been proposed to typify T_H determinants (40, 47, 52, 54) was present in all of the respective determinants. The specific I-A^d and I-E^d restriction motifs identified by Sette et al. (52, 54) had the highest success rate (Table 3) and were displayed by the determinants with the highest stimulatory potencies. The latter is probably a good reflection of the peptide's affinity for Ia. The generally increased content of the T_H determinant regions in basic charged amino acids and in some of the neutral hydrophobic amino acids (Ala, Phe, Met, and Val) appears to be a reflection of these motifs. Overall, these findings support the general notion that the structural requirements for T_H determinant function are not stringent.

A very surprising finding was that none of the 36 T_H clones recognized a determinant in the ectodomain of HA₂ (~170 amino acids) which makes up approximately one-third of the BHA molecule. Similarly, analysis of many T_H clones specific for the HA of the H3 subtype by two other groups (5, 12, 13, 37) failed to reveal a single clone with proven specificity for HA₂. These observations are in apparent contrast to two previous studies that reported strong T-cell responses to HA₂ in BALB/c mice immunized with influenza virus of the H3 subtype (3, 34). However, both of the latter studies were based on proliferation of mass cultures and may have suffered from difficulties in characterizing the proliferating cells or from an inadequate purity of the antigen preparation used for stimulation. Thus, the weight of current evidence speaks against HA₂ being a significant target structure for class II-restricted T cells. If so, what could account for the preferential recognition of HA₁ as opposed to the HA₂ ectodomain by T_H ?

First, this preference could reflect structural differences between these polypeptides which influence T_H determinant function. Differences in primary sequence are unlikely in view of the low stringency of sequence requirements for T_H determinants. Furthermore, no significant preference of T_H determinants for a certain secondary structure within HA₁ was evident in this analysis (Table 4). However, the large difference in the alpha-helical contents of HA₁ and HA₂ leaves open the possibility of a negative association between alpha-helical structure and the formation of T_H determinants: alpha helix makes up 6% of HA₁ and 53% of the HA₂ ectodomain (62a). This possibility would be in conflict with the amphipathicity model (40).

Second, HA₁ and HA₂ may be processed differently, at least when provided to the APC in the form of intact virus with cleaved HA, which was the case here for most priming regimens in vivo and for most restimulations in vitro prior to cloning or specificity analysis. It is well established that the HA undergoes a conformational change at pH ~5 (56), which corresponds to the acidity that the virus encounters upon uptake into endosomal vesicles of the APC. This conformational change activates fusion activity of the HA, which may eventually result in fusion between viral and endosomal membrane and release of viral nucleic acids and associated proteins into the cytoplasm. In addition, this conformational change makes, among others, a region of the N terminus of HA₁ highly susceptible to enzymatic cleavage (22, 31, 56). Since HA₁ is disulfide linked through Cys-4 at its N terminus to HA₂ (Fig. 5), an enzymatic cleavage in the N-terminal region of HA₁ would readily release a large C-terminal HA₁ fragment from HA₂ (22, 31, 56). The more intimate association of HA₂ with the viral lipid bilayer could

then result in different processing compared with the released and soluble HA₁ fragment. Such a possibility is supported by recent studies showing induction of class I-restricted cytotoxic T-cell responses following immunization with proteins or peptides incorporated into liposomes (21, 59), while free proteins or peptides typically induce T_H responses (10, 43). Differential processing could also result from intracellular acid-induced fusion between the viral and endosomal membrane, again leading to a more intimate association of HA₂ with this cellular membrane system. Related to this may be the observation that neuraminidase, the second major glycoprotein of influenza virus, is processed much more efficiently for T_H recognition following exposure of APC to purified neuraminidase than after exposure to intact virus (26); only in the latter case would a fusion-induced incorporation of neuraminidase into the endosomal membrane be expected to occur. This idea is not necessarily in conflict with the recent observation by Polydefkis et al. (45) that processing of human immunodeficiency virus type 1 gp120 for T_H recognition following endogenous synthesis required that gp120 (which is analogous to HA₁) remained covalently linked to gp41 (which, analogously to HA₂, anchors the glycoprotein into the viral/cellular membrane). The study did not exclude the possibility that this anchorage was merely required for effective recirculation of endogenously synthesized HIV glycoprotein through the endosomal compartment, thus providing some HIV glycoprotein to the cell for processing, albeit inefficiently.

A third possibility, proposed previously by Thomas and collaborators (5, 6, 13), is that T_H determinants of HA₁ are preferentially processed because HA₁ is preferentially recognized also by antibodies. The idea is based on the observations that (i) B cells are very effective in uptake and presentation of the specific antigens recognized by their surface immunoglobulin receptors (16, 18, 38, 46) and (ii) uptake of antigen via surface immunoglobulin receptors (or in the form of antigen-antibody complexes) may influence how the antigen is subsequently processed and which spectrum of T_H determinants will ultimately be presented for stimulation of T_H cells (19, 20, 39). In support of this view, Thomas and collaborators (5) presented evidence indicating that most T_H recognition sites in the H3 subtype HA were part of B-cell recognition sites. Although six of the T_H determinants identified here also contain at least one amino acid residue that is also part of a B-cell epitope, this appears to reflect a coincidental rather than causal relationship between B-cell and T_H determinants in the HA. Furthermore, we have shown previously (49, 50) that HA-specific B cells can process and present many viral T_H determinants other than HA, which also speaks against an obligate association between B- and T-cell recognition sites. In addition, the ability of influenza virus to adsorb efficiently to ubiquitous sialic acid-containing components present on the surfaces of all sorts of APC should make its effective uptake and presentation quite independent of HA-specific B cells (23).

Taken together, the data indicate that differential processing of an HA₁ fragment released from the lipid bound HA₂ by an endopeptidase in an acidic intracellular compartment is the most likely cause for the preferential recognition of HA₁ by T_H . However, further experiments are needed to confirm this. An important test for the differential processing may come from screening for T_H with specificity for the transmembrane region of HA₂. This region is recognized by class I-restricted cytotoxic T cells (9, 36) and, when used as a peptide for immunization, has been shown to induce peptide-reactive T_H (44). The prediction is that this determinant

would not be generated, or would be generated very inefficiently, upon natural processing of exogenously provided noninfectious virus.

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