Overlapping Cytotoxic T-Lymphocyte and B-Cell Antigenic Sites on the Influenza Virus H5 Hemagglutinin

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To define the recognition site of cytotoxic T lymphocytes (CTLs) on influenza virus H5 hemagglutinin (HA), an H5 HA-specific CTL clone was examined for the ability to recognize monoclonal antibody-selected HA variants of influenza virus A/Turkey/Ontario/7732/66 (H5N9). On the basis of ⁵¹Cr release assays with the variants, a CTL epitope was located near residue 168 of H5 HA. To define the epitope more precisely, a series of overlapping peptides corresponding to this region was synthesized and tested for CTL recognition. The minimum peptide recognized by the CTL clone encompassed residues 158 to 169 of H5 HA. Relative to the H3 HA three-dimensional structure, this CTL epitope is located near the distal tip of the HA molecule, also known as a major B-cell epitope on H3 HA. A single mutation at residue 168 (Lys to Glu) in the H5 HA variants abolished CTL recognition; this same amino acid was shown previously to be critical for B-cell recognition (M. Philpott, C. Hioe, M. Sheerar, and V. S. Hinshaw, *J. Virol.* 64:2941–2947, 1990). Additionally, mutations within this region of the HA molecule were associated with attenuation of the highly virulent A/Turkey/Ontario/7732/66 (H5N9) (M. Philpott, B. C. Easterday, and V. S. Hinshaw, *J. Virol.* 63:3453–3458, 1989). When tested for recognition of other H5 viruses, the CTL clone recognized the HA of A/Turkey/Ireland/1378/83 (H5N8) but not that of A/Chicken/Pennsylvania/1370/83 (H5N2), even though these viruses contain identical HA amino acid 158-to-169 sequences. These results suggest that differences outside the CTL epitope affected CTL recognition of the intact HA molecule. The H5 HA site defined in these studies is, therefore, important in both CTL and B-cell recognition, as well as the pathogenesis of the virus.

Influenza A virus infections induce a cytotoxic T-lymphocyte (CTL) response which is important in limiting viral replication and promoting viral clearance (24, 33). The CTL response to influenza A viruses is directed to a variety of viral proteins, including hemagglutinin (HA) (5, 32), nucleoprotein (38), matrix protein (14), neuraminidase (37), and polymerases (3). HA, a glycoprotein essential for viral attachment and entry, is an important target antigen for CTLs. Induction of CTLs specific for H1 (15, 21, 32), H2 (5), and H3 (31) HAs has been observed in response to influenza viruses A/Puerto Rico/8/34 (H1N1) and A/Japan/305/57 (H2N2) and H3 reassortant virus X-31, respectively. Our previous report (18) showed that CTLs from mice primed with avian influenza virus A/Turkey/Ontario/7732/66 (Ty/Ont) (H5N9) were predominantly HA specific and these CTLs played a role in protection during Ty/Ont infections.

The B-cell epitopes on HA have been well defined, and recently investigators have mapped T-cell epitopes on several HA subtypes. However, it is not clear whether B- and T-cell epitopes are typically located in the same regions on HA. On H1 HA, epitopes of class I-restricted CTLs (21) and class II-restricted T-helper cells (13, 19) have been identified in regions inside and outside the B-cell antigenic sites. In the H2 HA, two immunodominant regions were recognized by class I (*H-2^d*)-restricted CTLs (4, 6) and one of these epitopes was shown to overlap with a neutralizing antibody-binding site on H2 HA (30). In response to H3 HA, virus-specific CTLs (31) and T-helper cells (1, 7) were induced and

shown to recognize epitopes within the B-cell antigenic sites. However, Lamb et al. (23) identified a different T-helper cell epitope in an H3 HA region distinct from the B-cell sites. More studies on T- and B-cell epitopes of the different HA subtypes would reveal the prevalence of the antigenic sites shared by T and B cells. This information may contribute to a better understanding of antigen recognition by the humoral and cellular arms of the immune system.

To determine the CTL epitope on H5 HA, we examined the ability of a class II (*I-E^d*) major histocompatibility complex-restricted CTL clone (18) to recognize monoclonal antibody-selected Ty/Ont variants and synthetic peptides. From this study, we concluded that the minimal CTL epitope for this clone includes residues 158 to 169, which are located at the distal tip of the HA molecule. Within this epitope, a mutation at residue 168 (Lys to Glu) abolished CTL recognition; this same mutation was previously shown to affect the B-cell recognition and pathogenesis of Ty/Ont (27). Collectively, these studies suggest that a region at the distal tip of H5 HA induces protective B-cell and CTL responses to Ty/Ont and plays an important role in the biology of this highly virulent virus.

MATERIALS AND METHODS

Animals. Female BALB/c (*H-2^d*) mice were purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass., and used at 6 to 20 weeks of age.

Viruses. Influenza virus strains Ty/Ont, A/Turkey/California/Hurst-2/71, A/Chicken/Scotland/1/57 (Ck/Scot), and A/Chicken/Pennsylvania/1370/83 (Ck/Penn) were available from the virus repository at the School of Veterinary Medicine, University of Wisconsin, Madison. A/Turkey/Ireland/1378/83 (Ty/Ire) (20) was provided by R. G. Webster (St.

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Jude Children's Research Hospital, Memphis, Tenn.). Monoclonal antibody-selected variants of Ty/Ont have been previously described (26). The sequence data and mutations of the HA gene from each of the variants have been reported (27). These viruses were grown in the allantoic cavities of 10-day-old embryonated chicken eggs, harvested, and stored as infectious allantoic fluid at -70°C for use in ^{51}Cr release cytotoxicity assays.

Vaccinia virus recombinant H5Vac was prepared and generously provided by T. M. Chambers and R. G. Webster. The recombinant was prepared by inserting the H5 gene of Ty/Ire (20) into the thymidine kinase gene of vaccinia virus (10). Construction of the recombinant involved the use of plasmid pSC11 (9), provided to T. M. Chambers by B. Moss (National Institute of Allergy and Infectious Diseases, Bethesda, Md.). The H5Vac recombinant was grown and purified as previously described (18).

Cell lines. B-cell lymphoma line A20-1.11(*H-2^d*) (25), kindly given by T. Braciale (Department of Pathology, Washington University, St. Louis, Mo.), was maintained in Dulbecco modified Eagle minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.)–high glucose–high bicarbonate plus antibiotics–10% fetal bovine serum (HyClone Laboratories, Logan, Utah)–L-glutamine–nonessential amino acids. The LK35.2 (*H-2^{kxd}*) cell line is an A20.2J $\times$ B10.BR B-cell hybridoma (6) and was kindly provided by D. B. Thomas (National Institute for Medical Research, Mill Hill, London, Great Britain) and maintained in Dulbecco modified Eagle minimal essential medium supplemented with 10% fetal bovine serum, L-glutamine, nonessential amino acids, and antibiotics.

CTL clone and lines. CTL clone 3C5B4 was derived from BALB/c mice immunized with Ty/Ont. Generation, isolation, and characterization of the clone were previously described (18). Briefly, the clone was isolated by limiting dilution from a Ty/Ont-specific CTL line and then passaged weekly with A/Turkey/California/Hurst-2/71 (H5N2)-infected, irradiated, syngeneic spleen cells with a concanavalin A-stimulated rat splenocyte culture supernatant as a source of T-cell growth factors.

Peptides. Synthetic peptides corresponding to H5 HA sequences were produced on a semimanual solid-phase peptide synthesizer (RaMPS; DuPont, Wilmington, Del.). The purity of the crude product was assayed by using high-pressure liquid chromatography (HPLC) with a C18 reverse-phase column. Since all samples were shown to contain $>90\%$ of the desired product, the peptides were used in ^{51}Cr release cytotoxicity assays without further purification.

^{51}Cr release cytotoxicity assay. ^{51}Cr release assays were performed as previously described (18). Briefly, A20-1.11 and LK35.2 cells were labeled with ^{51}Cr and treated with an appropriate antigen (e.g., a peptide, influenza virus, or vaccinia virus). These target cells were then plated with T cells at designated ratios on 96-well microtiter plates. After 4 to 6 h of incubation at 37°C , the supernatant from each well was removed with a Skatron harvesting press (Skatron, Lier, Norway). The radioactivity of each sample was counted on an LKB1282 Compugamma (Pharmacia LKB Biotechnology, Gaithersburg, Md.). Results, presented as percent specific activity, were calculated as follows: [(test counts per minute – spontaneous counts per minute)/(total counts per minute – spontaneous counts per minute)] $\times$ 100. Spontaneous release and total ^{51}Cr counts were obtained from target cells incubated in medium alone and those disrupted with 5% Triton X-100, respectively; spontaneous counts per

TABLE 1. Recognition of Ty/Ont laboratory variants by CTL clone 3C5B4

Epitope group	Ty/Ont variant ^a	Amino acid no. (change)	% Sp act of clone 3C5B4 ^b
1	77B1v	168 (Lys to Glu)	0
	42C3v	168 (Lys to Glu), 198 (Ala to Thr)	<0
	67G1v	168 (Lys to Glu), 198 (Ala to Thr)	<0
	56G2v	205 (Arg to Ile)	49
2	4C9v	69 (Lys to Glu)	67
3	4F10v	52 (Glu to Lys), 198 (Ala to Thr)	49
4	76E1v	131 (Arg to Gln)	43
5	24B9v	157 (Ser to Pro)	52

^a Ty/Ont variants were selected with monoclonal antibodies to five B-cell epitopes of H5 HA (26, 27).

^b Specific activity of CTL clone 3C5B4 was assessed in ^{51}Cr cytotoxicity assays with A20-1.11 target cells infected with Ty/Ont variants at an effector/target ratio of 5:1. With the wild type, the specific activity of clone 3C5B4 was 55%.

minute ranged from 10 to 30% of the total count. Values for specific activity represent the mean counts per minute of triplicate samples. The standard deviation ($<5\%$) was omitted from tables and figures.

Three-dimensional HA structure analysis. Alignment of the H5 HA of Ty/Ont with the published H3 HA sequence (36) was done by first generating primary sequence alignment with the Best Fit program developed by the University of Wisconsin Genetics Computer Group, followed by three-dimensional alignment with an Evans and Sutherland PS390 interfaced with a MicroVax 2000 computer. Graphic output was generated on a Macintosh computer by using the Macinplot program generously provided by Tom Smith (Purdue University, West Lafayette, Ind.).

RESULTS

Isolation and characterization of *I-E^d*-restricted, CD8⁺ CTL clones specific for the H5 HA of Ty/Ont have been previously described (18). Since all isolated clones have the same antigenic specificity (C. E. Hioe and V. S. Hinshaw, unpublished data), one representative clone, designated 3C5B4, was chosen for further analyses.

CTL epitope mapping based on recognition of Ty/Ont monoclonal antibody-selected variants. To define CTL epitopes on H5 HA, we examined the ability of CTL clone 3C5B4 to recognize a panel of Ty/Ont variants selected with neutralizing monoclonal antibodies to five distinct B-cell epitopes of H5 HA (26). The HA gene of each variant had been previously sequenced to map five B-cell epitopes and also to identify point mutations involved in attenuation of virulent Ty/Ont (27). In ^{51}Cr cytotoxicity assays (Table 1), clone 3C5B4 lysed target cells (A20-1.11) infected with wild-type Ty/Ont and variants in four of the five previously defined B-cell groups. However, group 1 variants, i.e., 77B1v, 67G1v, and 42C3v, were not recognized. These three viruses shared a common point mutation at residue 168 (Lys to Glu) of H5 HA which previously had been identified as a major B-cell epitope (27). A second change at residue 198 (Ala to Thr) was found in both 67G1v and 42C3v but not in 77B1v. Since variant 4F10v, which was recognized by the CTL clone, also contains this mutation, we conclude that

FIG. 1. Overlapping peptides corresponding to the H5 HA amino acid 127-to-211 sequence of Ty/Ont and recognition of H5 HA peptides by CTL clone 3C5B4 in ^{51}Cr release assays. Target cells (A20-1.11) were treated with the peptides at a concentration of 25 $\mu\text{g}/\text{ml}$ and incubated with the CTL clone at an effector/target ratio of 8:1.

residue 198 was not involved in CTL recognition. These data suggest that HA residue 168 of Ty/Ont was essential for CTL recognition and that the region surrounding this amino acid comprised the CTL epitope recognized by clone 3C5B4.

CTL epitope mapping with synthetic peptides. To define precisely the epitope recognized by CTL clone 3C5B4, a series of overlapping peptides to the H5 HA region of Ty/Ont around residue 168 was made (Fig. 1). In ^{51}Cr cytotoxicity assays (Fig. 1), A20-1.11 target cells treated with peptide H5-p3 (amino acids 152 to 171) were specifically killed by clone 3C5B4 while cells treated with the other peptides were not. These data map the specificity of CTL clone 3C5B4 to the H5 HA amino acid 152-to-171 peptide of influenza virus Ty/Ont.

To determine the minimal sequence required for CTL recognition, a series of nested peptides within the amino acid 152-to-171 HA sequence was made and tested in cytotoxicity assays. The CTL clone recognized peptides H5-p3.2, H5-p3.3, H5-p3.4, and H5-p3.6, which encompassed an H5 HA sequence from residues 158 to 169 (Table 2). The other tested peptides lacked this segment and were not recognized. These results indicate that the CTL clone recognized

an epitope which included the HA amino acid 158-to-169 sequence of Ty/Ont.

The failure of the CTL clone to recognize Ty/Ont variants with a mutation at residue 168 (see above) suggested that this particular amino acid was essential for the CTL epitope. To confirm this hypothesis, a synthetic peptide corresponding to the HA amino acid 152-to-171 sequence of variant 77B1v (168 = Glu) was examined for CTL recognition. The cytotoxicity results (Fig. 2) showed that the clone did not recognize this variant peptide when tested at a concentration range of 15 to 125 $\mu\text{g}/\text{ml}$. In contrast, the analogous peptide with a wild-type sequence (168 = Lys) was recognized at a concentration as low as 4 $\mu\text{g}/\text{ml}$. These data confirm the importance of residue 168 in epitope recognition by CTL clone 3C5B4 and provide direct evidence that this single amino acid change within the CTL epitope was sufficient to abolish CTL recognition. The same mutation at 168 (Lys to Glu) has also been shown to affect B-cell recognition (27),

TABLE 2. Recognition of nested peptides within the H5 HA amino acid 152-to-171 sequence of Ty/Ont by CTL clone 3C5B4

Peptide ^a	Amino acid sequence	% Specific cytotoxicity of clone 3C5B4 ^b
H5-p3	152PYNGRSSFYRNVVWLIKNN171	56
H5-p3.2	154-----171	58
H5-p3.3	156-----171	54
H5-p3.4	158-----171	43
H5-p3.5	160-----171	6
H5-p3.6	152-----169	25
H5-p3.7	152-----167	7
H5-p3.8	152-----165	8
H5-p3.9	152-----163	6
H5-p3.10	152-----161	7

^a Peptides were tested at a concentration of 50 $\mu\text{g}/\text{ml}$, except for H5-p3.6, which was tested at a concentration of 1 $\mu\text{g}/\text{ml}$ because of toxicity.

^b The CTL clone was assayed in ^{51}Cr release assays with A20-1.11 target cells treated with the designated peptides at an effector/target ratio of 2:1.

FIG. 2. Recognition of peptide H5-p3 (H5 HA amino acid 152-to-171 sequence of wild-type Ty/Ont) and peptide H5-p3.1 (H5 HA amino acid 152-to-171 sequence of the 77B1v Ty/Ont variant) by CTL clone 3C5B4. The cytotoxicity of the CTL clone was assessed in ^{51}Cr release assays against LK35.2 target cells pulsed with various concentrations of H5 HA peptides at an effector/target ratio of 1:1.

FIG. 3. Location of the H5 HA amino acid 158-to-169 sequence encompassed in the epitope of CTL clone 3C5B4 projected onto a three-dimensional diagram of the influenza virus H3 HA monomer (36) and based on computer-generated alignment of the H5 and H3 HA sequences. The thicker line indicates the H5 HA amino acid 158-to-169 segment, which overlaps with a major B-cell epitope, designated site B (hatched region).

indicating that residue 168 was involved in both CTL and B-cell epitopes.

Localization of the CTL epitope on the three-dimensional HA structure. When the H5 HA amino acid sequence of Ty/Ont was aligned with that of H3 HA, we were able to model the location of the CTL epitope described above relative to the three-dimensional structure of H3 HA. The CTL epitope mapped close to the distal tip of the HA molecule (Fig. 3). This region has been established as a B-cell epitope, designated site B (hatched area), for influenza H3 viruses (35). Additionally, our previous studies with monoclonal antibody-selected Ty/Ont variants had suggested that the analogous region was also defined as a major B-cell epitope of H5 HA (27). This structural analysis supports our experimental data, suggesting that the CTL epitope of H5 HA, which is recognized by clone 3C5B4, overlaps with a major B-cell epitope.

CTL recognition of naturally occurring influenza H5 viruses. To determine whether clone 3C5B4 recognized different H5 HAs, naturally occurring H5 viruses were examined in cytotoxicity assays. The results (Table 3) showed that the CTL clone recognized Ty/Ont, Ty/Ire, and the H5 HA of Ty/Ire expressed by a vaccinia virus recombinant but

TABLE 3. Recognition of naturally occurring influenza H5 viruses by CTL clone 3C5B4

Virus	% Specific cytotoxicity for clone 3C5B4 ^a	HA amino acid 158-169 sequence
Ty/Ont/7732/66 (H5N9)	50	SFYRNVVWLIKK
Ty/Ire/1378/83 (H5N8)	39	—F—
Ty/Ire/1378/83 (H5Vac) ^b	26	—F—
Ck/Scot/1/57 (H5N1)	5	—L—
Ck/Penn/1370/83 (H5N2)	6	—F—

^a The CTL clone was assayed in ⁵¹Cr release assays with infected A20-1.11 target cells at an effector/target ratio of 5:1.

^b The H5 HA of Ty/Ire was expressed in a vaccinia virus recombinant.

failed to lyse A20-1.11 target cells infected with Ck/Scot or Ck/Penn. These data suggest that the clone was specific for the epitope expressed by Ty/Ont and Ty/Ire. To compare the HA sequences of these viruses, we obtained the sequence data for Ty/Ire and Ck/Penn from the GenBank Genetic Sequence Data Bank and used the previously published data for Ty/Ont (27) and Ck/Scot (12). A comparison of the HA amino acid 158-to-169 sequences from these viruses showed a high level of conservation, with the exception of residue 160, which is Tyr in Ty/Ont, Phe in Ty/Ire, and Leu in Ck/Scot. Thus, Phe and Tyr at residue 160 apparently did not alter CTL recognition but a Leu at this position abolished recognition by clone 3C5B4. To confirm these results, two peptides were made to the epitope sequences of Ty/Ire and Ck/Scot. The CTL clone recognized both peptides, as well as the peptide with the Ty/Ont sequence (data not shown), suggesting that changes at residue 160 (Tyr to Phe or Leu) do not affect CTL recognition.

Sequence comparison of Ck/Penn with Ty/Ire revealed no amino acid changes in the H5 HA amino acid 158-to-169 region, even though the CTL clone clearly showed differential recognition. The reason for this is not clear, but it is possible that specific residues outside the CTL epitope also affect processing of H5 HA or presentation of the epitope to the CTL clone.

DISCUSSION

Class II (*I-E^d*)-restricted CD8⁺ CTL clones specific for influenza virus H5 HA were generated from Ty/Ont-immunized BALB/c mice (18). To characterize the antigenic specificity for one of these CTLs, we mapped the epitope on the H5 HA sequence that was recognized by clone 3C5B4. CTL reactivity was assessed against Ty/Ont variants selected with neutralizing antibodies to H5 HA. On the basis of the recognition pattern and sequence comparison, we approximated the CTL epitope to a region around residue 168. To identify the epitope precisely, overlapping peptides corresponding to this region were made. The information provided by the variants allowed us to focus on one region of the HA and avoid the labor-intensive, costly method of synthesizing short peptides spanning the entire 566 amino acids (27) of H5 HA. By using these peptides, we determined that the epitope recognized by CTL clone 3C5B4 encompassed H5 HA residues 158 to 169 of Ty/Ont.

While most antibodies are known to recognize conformation-dependent epitopes on free antigens (11), T cells recognize predominantly processed antigens presented on the cell surface in the context of either class I (2, 14) or II (34) major histocompatibility complex molecules. Thus, it was generally expected that B and T cells would not recognize the

same site on a particular antigen. Our study, however, indicates that B- and T-cell epitopes are located in the same region of H5 HA. This observation correlates with previous reports showing that a dominant epitope recognized by CTL on H2 HA (30) and a number of recognition sites for T cells on H1 (19) and H3 (1, 7, 31) HAs are also associated with B-cell epitopes, indicating the presence of common antigenic regions for T and B cells on different HA subtypes.

Host antibody responses play a major role in the antigenic drift of influenza A viruses and HA in particular. This is evident from studies of naturally occurring viruses and monoclonal antibody-selected variants expressing residue changes that are clustered in common sites on HA molecules (8, 35). Our observations show that a single change at a particular antibody-defined epitope on H5 HA affected not only antibody binding but also CTL recognition. These and similar findings by other laboratories (1, 7, 30) reveal that B- and T-cell responses may share common antigenic sites on HA and that T cells, together with antibodies, may coexert immune pressure for antigenic drift in influenza virus HA glycoproteins. However, there is no direct evidence for immune selection of influenza virus variants in vivo by HA-specific T cells.

When recognition of the CTL epitope expressed by different H5 viruses was examined, we found that the HA of Ty/Ire contained the epitope recognized by the CTL clone; this was confirmed by CTL recognition of a synthetic peptide with the Ty/Ire epitope sequence. However, our clone failed to recognize Ck/Penn, which expresses an epitope with a primary sequence identical to that of Ty/Ire. In addition, the clone failed to recognize Ck/Scot, even though it recognized a peptide with the Ck/Scot epitope sequence. This suggests that the primary sequence of the epitope cannot be the only factor involved in its processing, presentation, and subsequent recognition. Possibly, residues distant from or flanking the epitope may affect the processing of the whole HA molecule and the presentation of this particular epitope to the CTL clone. Even though the HAs of Ck/Penn, Ty/Ire, and Ck/Scot are closely related (>87% amino acid identity) (12, 20), there are amino acid differences throughout their HAs. Thus, processing of the Ck/Penn and Ck/Scot HAs with secondary and tertiary structures distinct from the HA of Ty/Ire may yield antigenic fragments not recognized by the clone. This is supported by our results obtained with T-cell immunoblot assays. In these assays, the HA molecules were denatured, electrophoretically separated into its HA₁ and HA₂ subunits, and then blotted onto a nitrocellulose membrane for analysis in CTL proliferative assays. Our CTL clone did not recognize the blotted HA proteins (data not shown), suggesting that alteration in the tertiary structure of the HA antigen affected CTL recognition. Further studies will be needed to explore this possibility.

Rothbard and Taylor (28) have identified a motif commonly found in the primary sequences of many, but not all, T-cell epitopes. The motif consists of four (charged or glycine, hydrophobic, hydrophobic, and polar or glycine) or five (charged or glycine, hydrophobic, hydrophobic, hydrophobic or proline, and polar or glycine) amino acids and has been associated with T-cell epitopes in different proteins, including the H1 (17), H2 (30), and H3 (16, 22) HAs. In contrast to these epitopes, the CTL epitope on H5 HA lacks this pattern. More recently, Sette et al. (29) revealed a basic-X-basic motif specifically for *I-E*-restricted T-cell epitopes. Although our CTL clone is *I-E^d* restricted, its epitope does not contain this basic-X-basic pattern. Thus,

the CTL epitope defined in our studies did not fit either predicted motif.

A mutation at residue 168 (Lys to Glu) abolished epitope recognition by clone 3C5B4, indicating the critical role of this site in CTL recognition. However, on the basis of our observations, we do not know whether this loss of CTL recognition is due to the effect of the mutation on epitope interaction with the class II (*I-E^d*) major histocompatibility complex molecule or the T-cell receptor. To address this question, we are considering approaches to measure the affinity of the wild-type and mutant epitopes for the *I-E^d* gene products. Changes in this interaction may be responsible for the failure of the epitope to be correctly presented by *I-E^d* gene products to the CTL clone.

A question to be addressed regarding the CTL epitope is its immunogenicity, i.e., whether this epitope, important for both CTLs and B cells, can induce specific cellular and antibody responses in vivo. Our preliminary studies (Hioe and Hinshaw, unpublished data) revealed that the H5 HA amino acid 152-to-171 peptide (H5-p3), when inoculated subcutaneously in a single dose (50 µg per mouse) with complete Freund adjuvant, did not stimulate a T-cell proliferative or cytolytic response to Ty/Ont nor to the peptide itself. It is possible, however, that multiple administration of this peptide or coupling to a large protein immunogen would enhance the specific response to the peptide and, potentially, to the whole HA protein.

Our studies demonstrate that a class II (*I-E^d*)-restricted CD8⁺ CTL clone recognized an epitope on H5 HA which overlaps with a major B-cell antigenic site located on the distal tip of the HA molecule. Mutations at this site have previously been associated with attenuation of the highly virulent Ty/Ont (27), suggesting the importance of this site on the H5 HA molecule in the host response and biology of this virus. This information may prove helpful in developing effective vaccination strategies against influenza A viruses.

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