

# INFLUENZA VIRUS SITE RECOGNIZED BY A MURINE HELPER T CELL SPECIFIC FOR H1 STRAINS

## Localization to a Nine Amino Acid Sequence in the Hemagglutinin Molecule\*

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Recently, we characterized a monoclonal murine helper T cell ( $T_H$ )<sup>1</sup> line, Vir-2, which responds to the hemagglutinin (HA) of PR8 influenza virus and syngeneic antigen-presenting cells (APC) by specific proliferation, secretion of factors, and by delivering help for antibody production in vitro, or in nude mice after transfer and challenge by influenza infection (1). Vir-2 recognition of HA was approached by assaying proliferation on a panel of mutant viruses having known amino acid substitutions in the HA1 polypeptide. One of these mutants, which had a substitution of Glu→Lys at position 115 of the HA1, was not recognized by Vir-2.

This alteration could conceivably abrogate the stimulatory capacity of the HA molecule at different levels: for example, through long-range conformational effects or by conferring different susceptibility of the molecule to antigen processing, as well as by altering the specific residues contacted by the T cell receptor or by H-2 I region products (2–5). To further characterize the stimulatory determinant, biochemical localization of the recognized site was undertaken using purified HA and synthetic peptide homologues.

This paper identifies the site in HA recognized by Vir-2 cells. By identification of active cleavage fragments of HA, followed by synthesis of short peptides of the active segment, HA recognition by Vir-2 was delimited to a sequence of nine amino acids (positions 111–119) containing Glu<sub>115</sub>, which had mutated in the nonstimulatory variant. This sequence, which is located in the globular head region of the HA, is conserved in the currently sequenced human influenza viruses of the H1 subtype from 1934–1957 and 1977–1980, which explains the broad recognition of those viruses by Vir-2 cells.

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<sup>1</sup> Abbreviations used in this paper: APC, antigen-presenting cells; CNBr, cyanogen bromide; HA, hemagglutinin; HPLC, high-pressure liquid chromatography; NA, neuraminidase;  $T_H$ , helper T cell.

## Materials and Methods

*Vir-2 Cell Line and Proliferation Assays.* The HA-specific T<sub>H</sub> cell line Vir-2 was selected from the spleen of a BALB/c mouse immunized intraperitoneally with PR8 influenza virus, and cultured as previously described (1).

Proliferation assays were done in flat-bottomed microtiter wells (Linbro; Flow Laboratories, Inc., Hamden, CT) in a 0.2-ml final volume containing  $1 \times 10^4$ – $2 \times 10^4$  Vir-2 cells,  $4 \times 10^5$  irradiated spleen cells, and dilutions of antigen. At 48 h of culture, 0.2–0.5  $\mu$ Ci of [<sup>3</sup>H]thymidine (New England Nuclear, Boston, MA) (1 Ci =  $3.7 \times 10^{10}$  becquerels) was added in a 25- $\mu$ l volume, and cells were harvested 12–16 h later onto glass fiber disks (Flow Laboratories, Rockville, MD) for liquid scintillation counting.

*Viruses and HA Purification.* Type A influenza virus PR8 (A/Puerto Rico/8/34,H1N1), originally obtained from Mt. Sinai Hospital, New York, was grown in embryonated chicken eggs and titered by hemagglutination (6). Virus in allantoic fluid was used in proliferation assays without further purification.

The glycoproteins HA and neuraminidase (NA) were solubilized from purified virus by bromelain digestion according to Brand and Skehel (7) using a 1:10 enzyme/viral protein ratio. NA was removed from the sucrose density gradient-purified glycoprotein peak by the monoclonal anti-NA antibody H17/L17 (8) immobilized on protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) according to Gersten and Marchalonis (9). HA purity was assessed by sodium dodecyl sulfate (NaDodSO<sub>4</sub>) polyacrylamide gels (by the presence of only a single band in unreduced gels) and by radioimmunoassay (by the lack of binding to the preparation of a mixture of monoclonal antibodies specific for each of the NA, matrix protein, or nucleoprotein components of influenza).

*HA Polypeptide Separation and Cleavage Products.* Purified HA was separated into the heavy HA1 chain and the bromelain-shortened HA2 polypeptide by reduction and alkylation according to Skehel and Waterfield (10). Chains were isolated by chromatography on Sephacryl S-200 (Pharmacia Fine Chemicals) in 6 M urea, 0.2 M propionic acid.

The HA1 chain was cleaved at methionine residues by cyanogen bromide (CNBr) as recently described (11). Cleavage by CNBr at tryptophan residues was done according to Huang et al. (12). Fragments of HA1 were separated by high-pressure liquid chromatography (HPLC) size exclusion chromatography on an I-125 column (Waters Associates, Millipore Corp., Milford, MA), as described by Waterfield et al. (11).

*Peptide Synthesis.* Peptides were synthesized using the solid-phase method developed by Merrifield (13) or normal liquid-phase synthesis. To verify homogeneity and composition of active peptides, 100 mg of product was purified by HPLC using a preparative C18 ODS column (Waters Associates). Elution conditions were as follows: buffer A, 0.1% CF<sub>3</sub>COOH in H<sub>2</sub>O; buffer B, 0.1% CF<sub>3</sub>COOH in H<sub>2</sub>O/CH<sub>3</sub>CN, 30:70. The gradient was 25–90% buffer B for 50 min with a flow rate of 7 ml/min. After purification, 20 nmol of the peptide was subjected to automated Edman degradation as recently described (14), and shown to contain the complete expected sequence.

## Results

*Vir-2 Recognition of Purified HA and Cleavage Products.* Table I shows that Vir-2 cells proliferate in response to the HA of PR8 virus, provided as whole virus or as purified HA, in the presence of H-2<sup>d</sup> APC. Further, the stimulatory activity of the HA molecule was associated with the HA1 polypeptide chain. The small degree of stimulation observed with a relatively large amount of HA2 probably resulted from a slight contamination of the HA2 preparation with HA1. The isolated HA1 chain was efficiently recognized after reduction and alkylation, which suggests that the native HA conformation is not required for Vir-2 recognition.

This stimulatory activity was maintained after cleavage of the HA1 at Met or Met and Trp residues by CNBr (Table I). Determination of the cleavage fragment

TABLE I  
Region of the HA Recognized by Vir-2 Cells Resides Entirely in the HA1 Chain

Antigen	Form	Concentration	Uptake of [ <sup>3</sup> H]thymidine (cpm) by Vir-2 on APC of strains*	
			BALB/c (H-d <sup>d</sup> )	C3H/HeJ (H-2 <sup>k</sup> )
None	—		144	159
PR8 virus	Allantoic fluid	6 HAU/ml	13,584	119
HA	Bromelain-solubilized trimers	135 ng/ml	13,479	164
HA1 chain	Reduced and alkylated	80 ng/ml	13,341	134
		8 ng/ml	3,867	95
		0.8 ng/ml	297	120
HA2 chain	Reduced and alkylated	200 ng/ml	1,191	224
		20 ng/ml	169	192
HA1-CNBr-1	Cleaved at Met residues	300 ng/ml	8,284	96
		30 ng/ml	12,316	113
HA1-CNBr-2	Cleaved at Met and Trp residues	1,000 ng/ml	8,462	160
		100 ng/ml	8,082	115
		10 ng/ml	590	179

\* Data are mean counts per minute of four to six wells in replicate. Proliferation assayed as in Materials and Methods.

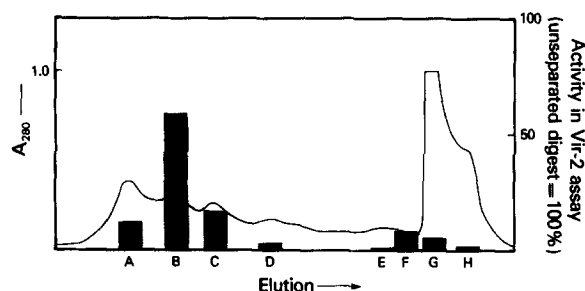


FIGURE 1. HPLC separation of fragments of PR8 HA1 after chemical cleavage at Trp residues by Vir-2 T<sub>H</sub> cells. Fragments of reduced and alkylated HA1 from CNBr digestion at Trp residues were separated on Waters I-125 sizing columns. Peaks, identified by A<sub>280</sub>, were pooled and tested in proliferation assays using Vir-2 cells. The histograms show stimulation of fractions relative to that obtained by the same dilution of unseparated HA1 CNBr digest.

that was recognized was approached as shown in Fig. 1. After CNBr cleavage at only Trp residues, the mixture of HA1 fragments was chromatographed on HPLC sizing columns. Pools of partially resolved peaks were tested for their ability to stimulate Vir-2 cells in a proliferation assay. Maximum activity was found in peak B (histogram, Fig. 1). N-terminal sequencing was then performed in order to define the peptide present in peak B. In each degradation step, two distinct residues were identified, which were compared with the known amino acid sequence of PR8 HA1 (15). The sequence of peptide B-1 was found to be: H<sub>2</sub>N-\_\_\_-Leu-Leu-Lys-Pro-Asp-. This corresponds to residues 232–237 and identifies the N-terminus of the C-terminal HA1 fragment (231–325) following Trp<sub>230</sub>. The N-terminal sequence of B-2 was: H<sub>2</sub>N-\_\_\_-Tyr-Ile-Val-Glu-, which matches residues 78–81 and identifies the peptide fragment 77–123. Considering

**Synthetic Peptides of the Recognized Sequence.** A series of peptide homologues ranging from 5 to 11 amino acids in length were synthesized covering positions within 109–120 (Table II). Vir-2 cells proliferated in response to the initial product, an undecapeptide of the region 109–119. Shorter peptides synthesized from either the C-terminal Lys<sub>119</sub> or Glu<sub>120</sub> were recognized by the T<sub>H</sub> only when extended in the amino terminal direction to include Phe<sub>111</sub>. By this analysis, recognition of HA by Vir-2 was delimited to the sequence of nine amino acids (Table II). Since the C-termini of the peptides were not extensively varied, the possibility remains that a somewhat shorter peptide may also be recognized by Vir-2 (see Discussion).

Some peptides, especially those covering positions 112–120 and 113–120, were not fully soluble in aqueous media, thereby necessitating studies in which the panel of peptides was solubilized in various detergents (NaDoSO<sub>4</sub>, sodium cholate, octyl glucoside). These detergents did not appreciably decrease Vir-2 proliferation in the presence of sufficient bovine serum albumin (0.05–2.5 g/100 ml) (not illustrated). None of these peptides were mitogenic for Vir-2, as observed by their failure to stimulate in the presence of H-2<sup>k</sup> APC.

**Efficiency of Stimulation: Whole HA vs. Smaller Products.** Although only picograms per milliliter of purified trimers of HA were required for threshold stimulation of Vir-2 in our proliferation assay, the synthetic peptides were needed in microgram per milliliter quantities. Table III shows that, on a molar basis, intact HA is  $10^6$ – $10^7$  times more efficient than the peptides, with the HA1 polypeptide and cleavage products distributed in between. Apparently, the longer

Synthetic peptide covering the segment 109-120 in PR8 HA1													Stock	Uptake of [ <sup>3</sup> H]thymidine (cpm) by Vir-2 on BALB/c APC: Dilution of peptide			Peptide (10 <sup>-3</sup> ) plus puri- fied HA*
109	110	111	112	113	114	115	116	117	118	119	120		10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>		
													<i>mg/ml</i>				
Ser-Ser-Phe-Glu-Arg-Phe-Glu-Ile-Phe-Pro-Lys													80	41,197	13,791	566	ND
Ile-Phe-Pro-Lys-Glu													1	381	513	617	56,554
Glu-Ile-Phe-Pro-Lys-Glu													1	741	743	1,229	54,705
Phe-Glu-Ile-Phe-Pro-Lys-Glu													1	412	560	840	57,391
Arg-Phe-Glu-Ile-Phe-Pro-Lys-Glu													1	226	658	760	56,215
Glu-Arg-Phe-Glu-Ile-Phe-Pro-Lys-Glu													1	387	813	668	54,238
Phe-Glu-Arg-Phe-Glu-Ile-Phe-Pro-Lys													5	54,182	29,868	6,526	ND
Cys-Phe-Glu-Arg-Phe-Glu-Ile-Phe-Pro-Lys-Glu													1	12,685	40,621	765	69,252

\* Uptake, HA alone is 57,438.

TABLE III  
*Comparative Efficiencies of Vir-2 Stimulation by HA and Smaller  
 Products, All Containing the Recognized Sequence*

Antigen	Amino acid length	Relative molar efficiency*
Peptide 109-119	11	1
Peptide 111-119	9	1
HA (CNBr at Trp)	47	3,500-7,000
HA (CNBr at Met)	226	120,000
HA1	325	200,000-400,000
HA	500	10 <sup>6</sup> -10 <sup>7</sup>

\* Calculated from the molar concentration of a given antigen sufficient for a threshold level of Vir-2 stimulation in a standard proliferation assay (see Materials and Methods), divided into the molar concentration required for similar stimulation by the 11 amino acid synthetic peptide (position 109-119).

the segment embodying the recognized sequence, the more efficiently it is recognized.

### Discussion

The HA site recognized by Vir-2 T<sub>H</sub> cells was localized to a sequence of nine amino acids (positions 111-119) in the PR8 influenza HA1 chain. Peptides of different lengths, synthesized from the C-terminal positions 119 or 120, were not recognized until extended according to the PR8 HA sequence to Phe<sub>111</sub>. Replacement at position 110 with the foreign Cys did not alter recognition of the stimulatory peptide. However, it is not known whether Vir-2 cells could also recognize peptides shortened further between positions 115 (known to be required from the mutant analysis; see reference 1) and residue 119, since C-termini were not extensively varied.

One complication in defining a recognition site by comparing responses to protein region homologues of different lengths is that these peptides can vary greatly in solubility. For example, two of the larger nonstimulatory octa- and nonapeptides were poorly soluble in water. Dissolving these peptides with detergents did not render them stimulatory for Vir-2. Elongation at the amino terminus simultaneously conferred water solubility and the ability to be recognized by Vir-2. It is not known whether these two properties are causally related, but it seems that inherent characteristics of certain homologues may have unknown effects upon their functioning in assays.

The nonapeptide 111-119 is the smallest sequence known thus far to be recognized by influenza virus-specific T<sub>H</sub> cells. Lamb et al. (16) identified an immunodominant peptide for HA-specific human T<sub>H</sub> cells covering the carboxy-terminal 25 amino acids of the H3 subtype HA1 chain. This peptide is in the stem region of the molecule, distant on the three-dimensional structure from the major sites in the globular head region where neutralizing antibodies are thought to bind in that strain (17). Smaller subunits of this 25 amino acid peptide, all having the same C-terminus, were found to be nonstimulatory for these T<sub>H</sub> clones (16).

Peptides as small as eight or nine amino acids are known to be recognized by T cells in other systems. Thomas et al. (18) have shown that T cells from guinea pigs immunized with the octapeptide angiotensin II specifically recognize that antigen in culture. Guinea pig T cells raised to insulin B chain are capable of responding to a synthetic eight amino acid segment of the antigenic region (4). Similarly, the main encephalitogenic determinant in guinea pigs for the T cell-associated disease experimental allergic encephalomyelitis is a nonapeptide of myelin basic protein (19). Therefore, our peptide ranks in size among the smallest known as T cell antigens.

Although both the intact HA molecule and active synthetic peptides are capable of eliciting maximal Vir-2 proliferation at optimal doses and have the same major histocompatibility complex restriction requirements, the stimulation efficiencies of these antigens differ greatly. Whereas  $\sim 20$  pg/ml ( $\sim 100$  fM concentration) suffices for the HA trimer, the threshold for stimulation with peptides was  $\sim 1$   $\mu$ g/ml (1  $\mu$ M). This difference of  $\sim 10^6$  appears mainly to reflect the extreme sensitivity of Vir-2 for the native HA, since the concentration thresholds for T cell stimulation in other systems are closer to the values we obtain with the undeca- and nonapeptides (2–4, 20–22). The basis of this sensitive response is not clear. Binding of the HA trimer to cell-surface sialic acid may focus the antigen on APC. Other possibilities could be different aggregation states of the antigens, or effects of glycosylation upon solubility or uptake. Thomas et al. (4) found that although an octapeptide of insulin was recognized by immune guinea pig T cells, it was less efficient in stimulating T cells than slightly larger (11 or 13 amino acid) overlapping peptides. The authors suggested that a conformation favorable for maximum T cell stimulation might be stabilized by the larger peptides. The possibility that additional nonspecific residues extraneous to the T cell recognition site may increase stimulatory efficiency is under study and could provide insight into the mechanism of antigen handling by APC.

The location of peptide 111–119 on the three-dimensional model of the H3 subtype HA (23) places it in the globular head region, where it would be on the exposed surface of the HA trimer. By laboratory selection of virus mutants with 15 mouse monoclonal anti-HA antibodies, only a single mutant was obtained with a residue change in that region, whereas numerous mutants were selected that possessed residue changes in HA1 segments that flank this peptide in the three-dimensional structure (15). This suggests that residues 111–119 do not constitute an important determinant for murine antibodies. In contrast, this segment seems to be the target of a commonly occurring murine T cell reactivity since  $T_H$  clones generated from two additional BALB/c mice have recently been found that recognize the 109–119 sequence of PR8 HA1 (J. L. Hurwitz, manuscript in preparation). These observations are similar to those reported for glucagon (24), lysozyme (20), angiotensin II (18), and myoglobin (21, 22), which indicates that B and T cells commonly focus onto different determinants on protein antigens.

This characteristic may explain our observation that the determinant identified by Vir-2 reactivity is present in all of the subtype H1 influenza viruses that have been tested so far (more than 15 strains, isolated originally within years 1934–1957 and 1977–1980) (1). No anti-HA antibodies exhibit such cross-reactivity,

which reflects the antigenic drift that presumably results from accumulation of point mutations in regions of the HA recognized by virus-neutralizing antibodies in the human population. Thus, as in BALB/c mice, the segment recognized by Vir-2 does not appear to be an important target structure for human neutralizing antibodies. This raises the question of whether, in an analogous way, the region is a significant determinant for human T<sub>H</sub>. It should be noted in this context that Lamb et al. (16) obtained good levels of proliferation by human peripheral blood lymphocytes in response to a synthetic peptide covering positions 105–140 of the subtype H3 HA molecule. We are currently studying recognition of the H1 HA by donor human T lymphocytes.

### Summary

The functional helper T cell line Vir-2, derived from a PR8 (H1N1) influenza virus-immunized BALB/c mouse, proliferates in response to syngeneic antigen-presenting cells and naturally occurring strains of subtype H1 human influenza virus from 1934–1957 and 1977–1980 isolates. A conserved region of the hemagglutinin molecule around amino acid position 115 in the heavy chain (HA1) was implicated as being important in this recognition by the lack of stimulatory activity associated with a glutamic acid to lysine substitution at position 115 in the laboratory mutant RV6, derived from wild-type PR8. Characterization of the stimulatory determinant on the wild-type hemagglutinin molecule was then undertaken using cleavage products and synthetic peptides. Vir-2 cells recognized the reduced and alkylated purified HA1 of PR8 virus, and this reactivity was retained after cleavage at methionine and tryptophan residues. High-pressure liquid chromatography separation of cleavage fragments indicated that a short sequence of the HA1 containing residue 115 was being recognized. This recognition was localized to a nine amino acid segment (positions 111–119) by assaying stimulation with synthetic peptide homologues of different lengths from that region. As with native hemagglutinin, Vir-2 cells responded to active peptides when presented by H-2<sup>d</sup> but not H-2<sup>k</sup> antigen-presenting cells.

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