

ON THE ROLE OF THE TRANSMEMBRANE ANCHOR  
SEQUENCE OF INFLUENZA HEMAGGLUTININ  
IN TARGET CELL RECOGNITION BY CLASS I  
MHC-RESTRICTED, HEMAGGLUTININ-SPECIFIC  
CYTOLYTIC T LYMPHOCYTES

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Foreign antigenic moieties are recognized by T lymphocytes in association with products of the MHC. Both class I MHC products (e.g., H-2 K/LD locus products in the mouse) and class II MHC products (e.g., murine H-2 I locus products) restrict T lymphocyte recognition of antigen (1). The mechanism by which antigen and MHC products are presented to T lymphocytes is not completely understood. Recently, however, studies by Babbit et al. (2) and Buus et al. (3) have indicated that a direct interaction between class II MHC products and processed forms of foreign antigens may constitute a critical step in the formation of the antigen moiety recognized by class II MHC-restricted T lymphocytes. Although the pathways of antigen presentation to class I and class II MHC-restricted T lymphocytes appear to be distinctly different (4), there is evidence that class I MHC-restricted T lymphocytes may also recognize processed forms of foreign antigens (5–10).

In this report we have examined the recognition of the type A influenza virus hemagglutinin (HA)<sup>1</sup> by class I MHC-restricted CTL with particular emphasis on the role of the hydrophobic transmembrane anchor sequence in the formation of the HA antigenic structure necessary for target cell recognition by H-2K<sup>d</sup>-restricted HA-specific CTL. We have observed that the product of a truncated HA gene that lacks the sequence corresponding to the hydrophobic anchor and cytoplasmic domains of the A/JAP/305/57 HA can be recognized by a majority of H-2K<sup>d</sup>-restricted HA-specific CTL. Further, transmembrane sequence itself appears to contain amino acid residues that make up antigenic epitopes recognized by H2N2 subtype-specific and crossreactive HA-specific CTL clones. The

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<sup>1</sup> *Abbreviations used in this paper:* DME, Dulbecco's modified minimal essential medium; HA, influenza hemagglutinin.

implications of these observations for viral antigen presentation in class I MHC CTL recognition are discussed.

### Materials and Methods

**Viruses.** Influenza virus strains A/JAP/57 (A/Japan/305/57 [H2N2]), and B/Lee were grown in the allantoic cavity of 10-d-old embryonated chicken eggs and stored as infectious allantoic fluid as previously described (11). SV40 virus stocks were produced as described by Doyle et al. (12). A recombinant vaccinia virus containing the A/JAP/57 gene (13, 14) was kindly provided by Dr. Bernard Moss. (National Institutes of Health, Bethesda, MD.)

**Cell Lines.** Low-passage CV-1 monkey cells were obtained from the American Type Culture Collection (Rockville, MD) and were passaged and maintained in Dulbecco's Modified Minimal Essential Medium (DME) supplemented with 10% (vol/vol) FCS. CVKD-10.6 cells, a neomycin-resistant derivative of the CV-1 cell line that expresses the murine H-2K<sup>d</sup> gene product, were maintained in the above medium further supplemented with the antibiotic G418 (Gibco Laboratories, Grand Island, NY) at a final concentration of 400 µg/ml. The protocols for maintenance of P815 cells in DME plus 10% FCS and of parental L cells (line K2) or transfected L cells expressing the K<sup>d</sup> gene product in (line K7-74) HAT selection medium have been described previously (15, 16).

**Recombinant SV40 Expression Vectors.** The construction and characterization of SV40-based late replacement vectors designed to express the wild-type and mutant A/JAP/57 HA molecules shown in Figure 2 have been described previously. The recombinant viruses used to express the various HA species were as follows: SVEHA3 [wild-type HA (17)]; SVEHA20-A<sup>-</sup> (anchor-negative HA [18]); SVEHA-G and SVEHA-gC (chimeric HAs containing the transmembrane and cytoplasmic domains of the VSV G glycoprotein and the HSV-1 C glycoprotein, respectively [19]). High-titer, second-passage (P2) virus stocks were generated as described previously (12), but the following modified protocol was used to infect CVKD-10.6 cells. Monolayers of exponentially growing cells were harvested by trypsinization and washed with DME containing 10% FCS. Aliquots of  $9 \times 10^5$  cells were resuspended in 100 µl of undiluted P2 virus stocks, incubated on ice for 10 min and then at 37°C for 60 min. After the addition of 5 ml of DME plus 10% FCS, the cells were plated in 60-mm culture dishes or 75-cm<sup>2</sup> flasks and incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> for 48–72 h before assay for expression of HA.

**Isolation of Monkey CV-1 Cells that Express the Murine K<sup>d</sup> Gene Product.** CV-1 cell transformants that express the product of the K<sup>d</sup> gene were established by cotransfection with DNA corresponding to this gene segment together with the bacterial gene that encodes resistance to the antibiotic G418. Antibiotic-resistant cell colonies were isolated and analyzed for expression of the K<sup>d</sup> gene product by RIA (15). Several positive cell lines were then tested for cell surface expression of the K<sup>d</sup> product by flow cytometry using a murine mAb (DO4) as described previously (20, 21). From the cell line expressing the highest level of K<sup>d</sup>, the brightest 10% of cells were sorted, grown up, and subjected to sequential selection for K<sup>d</sup> expression. Six sequential sorts yielded the cell line CVKD-10.6. Subsequent selection of K<sup>d</sup>-expressing cells yielded no further enhancement of expression. Cloned cell lines derived from CVKD-10.6 showed no greater stability or magnitude of K<sup>d</sup> expression than the parental line. Cell populations derived from each sequential sort were tested as target cells for K<sup>d</sup>-restricted CTL. For each sort the efficiency of target cell recognition was directly proportionated to K<sup>d</sup> expression as determined by fluorescence intensity.

**Synthetic Peptides.** A synthetic 23-amino-acid oligopeptide corresponding to the amino acid sequence from the anchor region of the A/JAP/57 HA (see Fig. 5) was produced on an automated solid-phase peptide synthesizer from Applied Biosystems, Inc. (Foster City, CA). This hydrophobic peptide was solubilized in 100% DMSO at a concentration of 5 mg/ml and serially diluted into DME without serum to a concentration of 5 µg/ml for use in cytotoxicity assays. Control experiments showed that the residual DMSO present in the peptide preparation used in cytotoxicity assays (0.025–0.00025%) had no effect on target cell stability or on the specificity or function of cloned CTL. The unfractionated

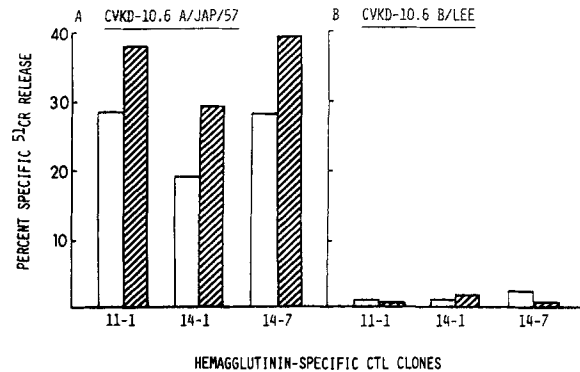


FIGURE 1. Recognition of influenza virus-infected CVKD-10.6 cells by murine K<sup>d</sup>-restricted CTL clones. Target cells were infected with A/JAP/57 or B/Lee viruses and tested for their susceptibility to lysis by three HA-specific CTL clones in a standard 6-h <sup>51</sup>Cr-release assay. The clones were used 3 d after subculture. Spontaneous release from target cells was <20% of total release during the assay period. E/T cell ratios used in the assays: □, 5:1; ▨, 20:1.

peptide was ~50% pure as estimated from percentage composition of individual amino acid residues.

*T Lymphocyte Clones.* The cloned T lymphocytes used in this assay were derived from BALB/c and CB6F<sub>1</sub> mice. They are all restricted by H-2K<sup>d</sup> and directed to the A/JAP/57 HA. Their establishment, maintenance, and antigenic fine specificity have been detailed in recent publications (14, 16).

*Assays for Cell-mediated cytotoxicity.* P815 cells, transfected L cells, and CV-1 cells expressing the K<sup>d</sup> gene product were used as targets in standard <sup>51</sup>Cr-release assays. These assays were carried out essentially as described previously (4). For assays using synthetic peptides, the anchor peptide was diluted in tissue culture medium to the appropriate concentration and added to a mixture of cloned CTL and target cells in 96-well microtiter plates. For assays using CVKD-10.6 cells infected with SV40 late replacement vectors as targets, cells were infected in suspension before plating and incubated for 48–72 h at 37°C to allow maximum HA expression before <sup>51</sup>Cr labeling. Cytotoxicity assays were carried out for 4–6 h as described (11), and the resultant data were analyzed exactly as described in detail elsewhere (4, 11).

## Results

*Antigen-specific Recognition by Murine CTL of Monkey CV-1 Cells Displaying the Murine K<sup>d</sup> Gene Product.* We have previously (20) used transfected L cells that express the influenza HA gene from a plasmid-based vector to show that the HA alone, in the absence of other influenza virus-encoded proteins, can be recognized by both strain-specific and crossreactive antiinfluenza CTL. However, the efficiency of CTL lysis of the transfected cell line was less than optimum as a result of the low level of expression of HA. To alleviate this problem and also to allow testing as target antigens, the full range of HA mutants that have been cloned into SV40-based vectors (reviewed in reference 19), we decided to generate a monkey cell line that would be both permissive for infection by SV40 and also histocompatible for lysis by murine class I MHC-restricted CTL. To this end, we have introduced the murine H-2K<sup>d</sup> gene into CV-1 cells under selection for neomycin resistance (Materials and Methods). Cells that were resistant to the G418 antibiotic were further selected for cell surface expression of the K<sup>d</sup> molecule by flow cytofluorometry. Several K<sup>d</sup>-expressing CV-1 lines were isolated by this protocol, including the CVKD-10.6 line used here. As demonstrated in Fig. 1, this K<sup>d</sup>-expressing cell line was recognized and destroyed by influenza-specific murine class I MHC-restricted CTL in an antigen-specific fashion. The three K<sup>d</sup>-restricted CTL clones, 11-1, 14-1, and 14-7, which recognize the

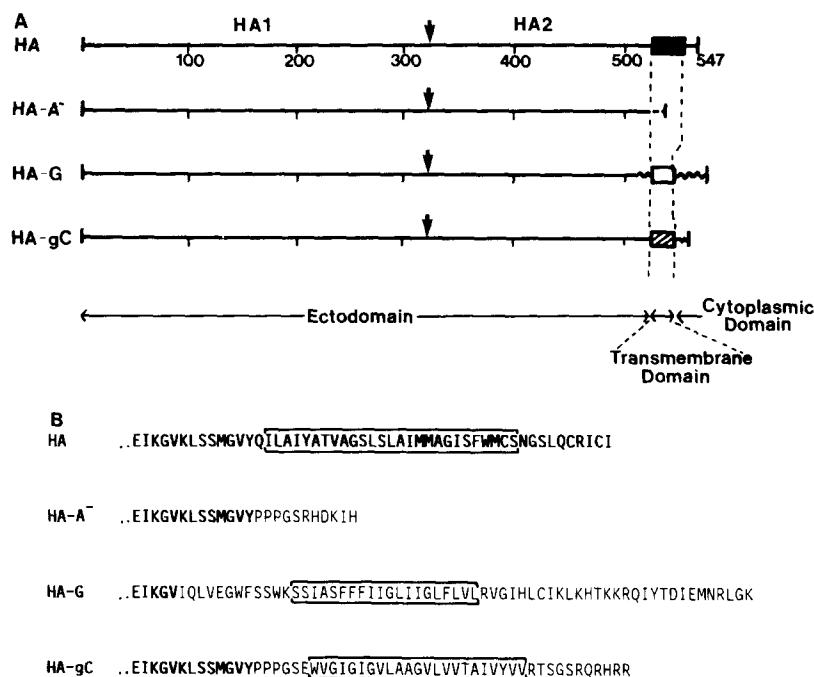


FIGURE 2. Comparison of the structures of wild-type, anchor-negative, and chimeric forms of HA. (A) A schematic diagram of the various HA polypeptides. Within the large NH<sub>2</sub>-terminal ectodomain of HA lies the trypsin-sensitive site (*arrowhead*) at which the precursor molecule can be cleaved into HA1 and HA2 subunits. The mutant proteins differ from the wild-type at the COOH-terminus; the transmembrane and cytoplasmic domains are absent (HA-A<sup>-</sup>) or have been substituted by the analogous regions of the VSV G glycoprotein (HA-G) or the HSV-1 C glycoprotein (HA-gC). (B) The COOH-terminal amino acid sequences of the wild-type and mutant HA glycoproteins (18).

A/JAP/57 HA (14), efficiently lyse A/JAP/57-infected, but not influenza B/Lee-infected CVKD-10.6 cells. A/JAP/57-infected parental CV-1 cells were not susceptible to lysis by these murine CTL clones (see Table IV).

*CTL Recognition of Anchor-negative HA.* We first chose to examine class I MHC-restricted CTL recognition of a secreted form of HA that lacks the hydrophobic transmembrane domain that normally anchors the protein in the lipid bilayer. A gene encoding this anchor-negative HA has previously been generated by deletion of the nucleotide sequence corresponding to the membrane-spanning and cytoplasmic portions of the HA molecule (18). To facilitate incorporation of the mutant gene into the expression vector, a short synthetic oligonucleotide sequence coding for a series of charged amino acids was added at the 3'-end of the truncated cDNA. The COOH-terminal sequence of the mutant HA is shown in Fig. 2. This truncated HA gene has been previously shown to be efficiently expressed in CV-1 cells using the SVEHA20-A<sup>-</sup> late replacement vector (18).

The results presented in Table I show that two HA-specific CTL clones, 11-1 and 14-1, recognize CVKD-10.6 cells infected with the SVEHA20-A<sup>-</sup> recombinant virus and do so with an efficiency comparable to that of CVKD-10.6 cells

TABLE I  
*CTL Recognition of the Products of Anchor-positive and Anchor-negative HA Genes*

CTL clone	E/T ratio	Percent specific <sup>51</sup> Cr release from infected CVKD-10.6 target cells*				
		A/JAP/57 <sup>‡</sup>	SVHA-A <sup>+</sup>	SVHA-A <sup>-</sup>	VV(HA)	B/LEE
11-1	5:1	16	6	10	23	0.1
	20:1	52	27	35	65	0.4
14-1	5:1	24	9	15	41	0.8
	20:1	37	13	20	58	0.3
14-7	5:1	20	12	0.1	36	0.3
	20:1	31	16	1.0	53	0.5

\* CTL clones were tested for lytic activity in a 6-h <sup>51</sup>Cr-release assay on CVKD-10.6 cells infected with either the indicated influenza strain (A/JAP/57, B/Lee), a recombination vaccinia virus containing the A/JAP/57 HA gene [VV(HA)], the SVEHA3 late replacement vector containing the intact A/JAP/57 HA gene (SVHA-A<sup>+</sup>), or the SVEHA20-A<sup>-</sup> vector containing the HA gene lacking the sequences encoding the transmembrane anchor (SVHA-A<sup>-</sup>).

<sup>‡</sup> Values are the mean of triplicate wells; SEM were <5% of mean values and are omitted. Spontaneous release from all targets was less than 20% of total incorporation. 10<sup>4</sup> target cells were added per well.

infected with the SVEHA3 virus that expresses the wild-type A/JAP/57 HA. In contrast, although clone 14-7 recognizes the parental HA gene product expressed either after infection with A/JAP/57 influenza virus or after infection with recombinant SVEHA3 or vaccinia virus (VV-HA) expression vectors containing the wild-type HA gene sequence, it fails to recognize the product of the truncated anchor-negative HA gene.

It is noteworthy that while HA was readily demonstrable on the surface of CVKD-10.6 cells infected with A/JAP/57 virus or the SVEHA3 vector containing the parental HA gene, flow cytometric analyses of cells infected with the SVEHA20-A<sup>-</sup> vector failed to reveal any cell surface HA (Fig. 3). This result is consistent with our previous observations that the anchor-negative HA is not anchored in the lipid bilayer and is not detectable on the surface of infected CV-1 cell as an integral membrane protein (18). Rather, this mutant protein is synthesized in the rough endoplasmic reticulum, transported through the Golgi apparatus and secreted into the medium overlying the infected cells. Thus, the ability of certain class I MHC-restricted CTL to recognize cells expressing this secreted HA molecule strongly suggests that display of HA as an integral membrane constituent is not a necessary requirement for class I MHC-restricted CTL recognition.

CTL clones 11-1, 14-1, and 14-7 are all Lyt-2<sup>+</sup> (16), and as demonstrated above and elsewhere (14, 16), are HA specific and K<sup>d</sup> restricted. However, clones 11-1 and 14-1, which recognize the anchor-negative HA, are directed to an epitope shared by the A/JAP/57 and serologically closely related HAs of the H2N2 subtype (14). On the other hand, clone 14-7, which fails to recognize the anchor-negative form of the HA, is more broadly crossreactive within the subtype and recognizes an HA epitope shared by most viruses of the H2N2 subtype (14, 16). In view of this difference in fine specificity between these CTL clones, it was of interest to examine recognition of the anchor-negative HA by additional HA-specific CTL clones. The results of this analysis for seven such clones are

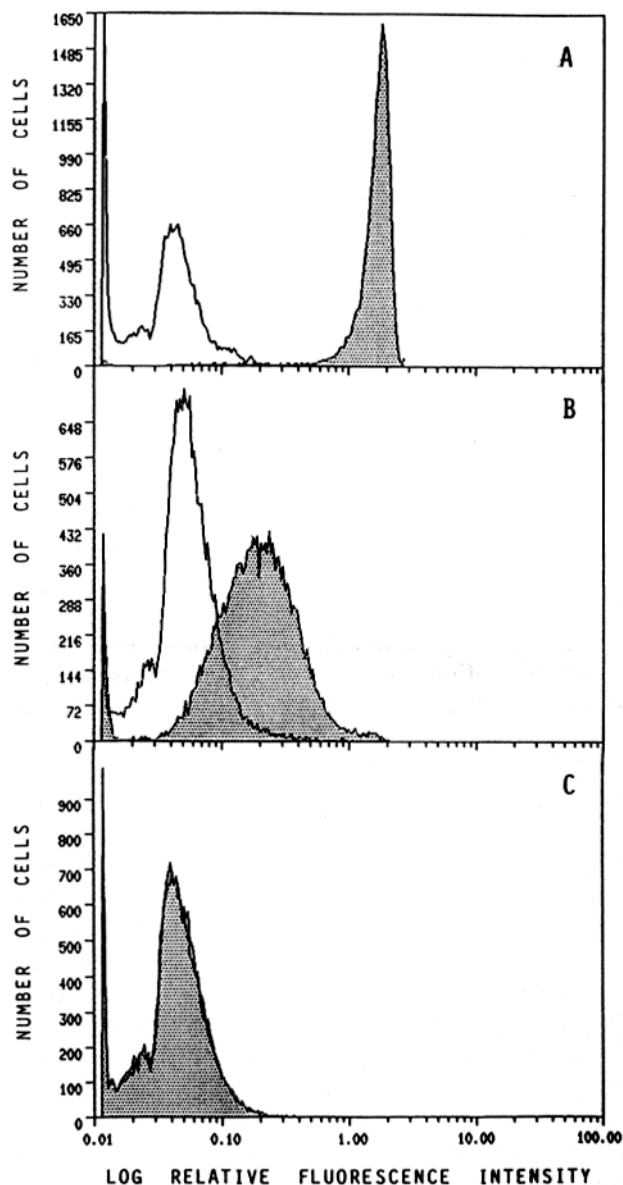


FIGURE 3. Flow cytometric analysis of the expression of HA glycoproteins on the cell surface of CVKD-10.6 cells. CVKD-10.6 cells were infected with (A) A/JAP/57 influenza virus, or (B) with SV40-HA recombinant viruses encoding the wild-type A/JAP/57 HA (SVEHA3), (C) or the anchor-negative HA (SVEHA20-A<sup>-</sup>). Shaded curves indicate the fluorescence intensity profile of infected cells treated with anti-HA mAb and fluoresceinated secondary anti-mouse Ig antibody. Open curves describe profiles obtained with secondary antibody alone.

presented in Table II. Five of these clones recognize target cells expressing the anchor-negative HA, albeit with varying efficiency. As reported by us elsewhere (14), these clones also recognize strain-specific HA epitopes expressed on the A/JAP/57 and closely related HAs. In contrast, two other HA-specific clones, A-4 and C-1, fail to recognize the anchor-negative HA. Clone A-1, like clone 14-7, is subtype specific, while clone C-1 recognizes an HA epitope shared by HAs of the H1N1 and H2N2 subtypes (14).

*CTL Recognition of Chimeric HA Genes Containing Unrelated Anchor Sequences.* There are two possible explanations for the lack of recognition of the

TABLE II  
*Anchor-negative Hemagglutinin Recognition and CTL Fine Specificity*

CTL clone	Percent specific <sup>51</sup> Cr release from infected CVKD-10.6 targets*			
	A/JAP/57†	SVHA-A <sup>+</sup>	SVHA-A <sup>-</sup>	B/LEE
17-2	36	13	9	0.2
17-4	38	13	10	0
40-1	44	38	34	1
40-2	27	22	23	0.2
40-4	33	31	27	0.5
A-4	17	10	1	0
C-1	29	20	1	0.7

\* As in Table I. E/T cell ratio was 20:1 in all cases.

† As in Table I.

anchor-negative HA by clones 14-7, A-4, and C-1. First, these clones could recognize epitopes encoded by the nucleotide sequence corresponding to the deleted transmembrane or cytoplasmic domains of the HA polypeptide (Fig. 2). Alternatively, the tertiary structure of the anchor-negative HA product might be altered as a result of the lack of membrane anchorage, resulting in selective loss of conformational epitopes recognized by these three HA-specific CTL clones. To examine this latter possibility, we analyzed the recognition of cells expressing chimeric HA gene constructs in which the sequences encoding the transmembrane and cytoplasmic domains of HA have been replaced by the corresponding domains from either the HSV-1 glycoprotein C (SVHA-gC) or the VSV G protein (SVHA-G). Previous experiments (19) have demonstrated that these chimeric proteins are anchored in the lipid bilayer and transported to the cell surface where they are displayed in an antigenically and biologically active form. The structures of these molecules are shown in Fig. 2. The results presented in Table III demonstrate that clones 14-7, A-4, and C-1 fail to recognize CVKD-10.6 cells expressing the chimeric HA gene products, indicating that recognition by these clones is not reestablished by anchoring the HA ectodomain in the plasma membrane. Three other HA-specific clones, 11-1, 40-1, and 40-2, which recognize the parental HA gene product and the anchor-negative HA, do recognize the products of the chimeric HA constructs with comparable efficiency.

*CTL Recognition of a Synthetic Peptide Corresponding to the Anchor Domain of the HA.* The results described above support the view that the three clones that fail to recognize the anchor-negative HA might be directed to sequences located within the deleted transmembrane and/or cytoplasmic domains of the A/JAP/57 HA. To address this possibility directly, we synthesized a synthetic peptide corresponding to 23 amino acid residues encompassing the less-conserved portion of the transmembrane region of the A/JAP/57 HA (Fig. 4) and examined the capacity of a panel of K<sup>d</sup>-restricted, HA-specific CTL clones of different fine specificity to recognize target cells sensitized with this peptide. Choice of the peptide was made after consideration of the evidence that although clones 14-7 and A-4 both recognize an epitope (or epitopes) contained within the transmembrane and cytoplasmic domains of the A/JAP/57 HA, they do not recognize cells

TABLE III  
CTL Recognition of Chimeric HA Gene Products Containing Unrelated Anchor Sequences

CTL clone	E/T ratio	Percent specific <sup>51</sup> Cr release from infected CVKD-10.6 target cells*				
		A/JAP/57 <sup>‡</sup>	SVHA-A <sup>+</sup>	SVHA-A <sup>-</sup>	SVHA-gC	SVHA-G
11-1	5:1	32	17	19	26	21
	20:1	51	25	28	37	31
40-1	5:1	49	45	40	50	41
	20:1	75	68	66	74	67
40-2	5:1	35	31	27	38	33
	20:1	61	53	56	62	56
14-7	5:1	28	15	0.7	0.6	0.1
	20:1	53	25	1	2	1
A-4	5:1	21	10	0.5	0.2	0
	20:1	43	17	1	1	1
C-1	5:1	27	18	1	2	1
	20:1	43	26	2	2	1

\* As in Table I. SVHA-gC and SVHA-G represent SV40 vectors containing chimeric gene sequences encoding the extracellular portion of the A/JAP/57 HA linked to the membrane-spanning and cytoplasmic regions of the herpes simplex I gC and VSV-G proteins. (See Materials and Methods and Fig. 2.)

‡ As in Table I.

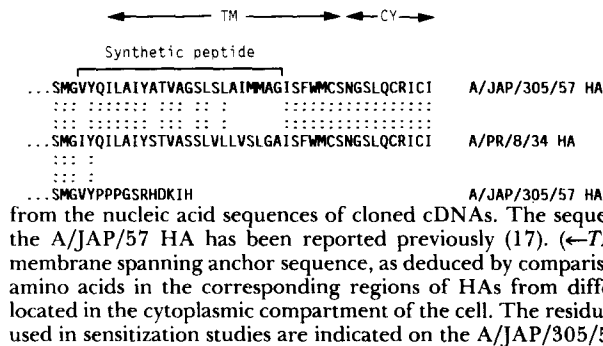


FIGURE 4. Comparison of the amino acid sequences of the transmembrane and cytoplasmic domains of the A/JAP/57 and A/PR/8 HA glycoproteins. The COOH-terminal sequences of the A/JAP/305/57 and A/PR/8/34 HA glycoproteins were deduced from the nucleic acid sequences of cloned cDNAs. The sequence of the anchor-negative mutant of the A/JAP/57 HA has been reported previously (17). (←TM→) The residues encompassing the membrane spanning anchor sequence, as deduced by comparison of the positions of charged or polar amino acids in the corresponding regions of HAs from different virus strains. (←CY→) Residues located in the cytoplasmic compartment of the cell. The residues represented in the synthetic peptide used in sensitization studies are indicated on the A/JAP/305/57 HA sequence.

expressing the A/PR8/34 HA. Comparison of the amino acid sequences of these two HAs (Fig. 4) shows that the cytoplasmic tail and the COOH-terminal portion of the transmembrane domain are totally conserved so that sequence divergence is evident only within the remainder of the transmembrane region. The peptide was designed to correspond to this less-conserved portion of the A/JAP/57 HA sequence.

The results shown in Fig. 5 indicate that nine CTL clones that recognize CVKD-10.6 cells expressing the product of the anchor-negative gene construct all fail to recognize target cells sensitized with the anchor peptide. However, the three clones (14-7, A-4, and C-1) that failed to recognize the anchor-negative HA product efficiently recognized peptide-treated target cells. Sensitization of target cells with the synthetic anchor peptide was dose dependent. The results presented in Fig. 6 show that sensitization was achieved at peptide concentrations as low as 12.5 ng/ml (4.5 nM) and was optimum at peptide concentrations 10–100-fold higher. Furthermore, anchor-specific CTL clones recognize target cells treated with the anchor peptide in an MHC-restricted fashion (Table IV). The



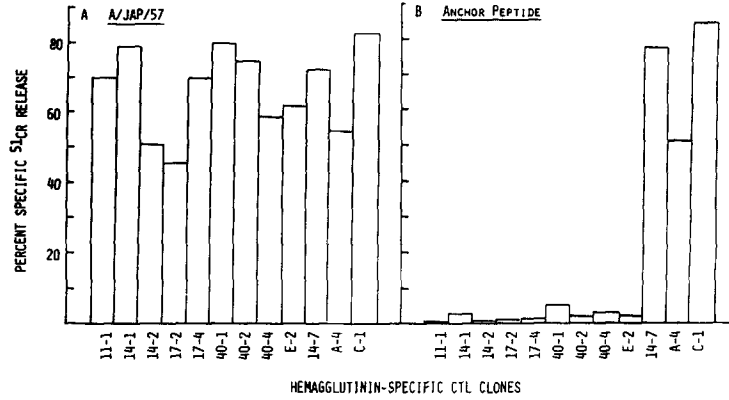


FIGURE 5. Specific sensitization of target cells by a synthetic anchor peptide. P815 cells (H-2<sup>d</sup>) were screened for lysis by the indicated HA-specific CTL clones in the presence of the synthetic anchor peptide. The E/T ratio was 5:1. The synthetic peptide was present throughout the course of the 6-h assay at a concentration of 1,250 ng/ml.

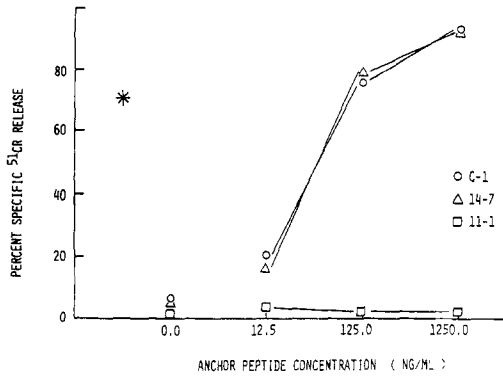


FIGURE 6. Dose dependence of target cell sensitization by the synthetic anchor peptide. The E/T cell ratio for the indicated anchor-specific CTL clones (C-1 and 14-7) is 5:1. P815 target cells were incubated with CTL in the presence of peptide at the indicated concentration. Clone 11-1 is an HA-specific K<sup>d</sup>-restricted clone that recognizes the anchor-negative HA. Asterisk indicates level of lysis of A/JAP/57-infected cells by CTL clone 11-1.

two anchor-specific CTL clones, 14-7 and C-1, recognized both the transfected L cells (cell line K7-74) expressing the K<sup>d</sup> gene product as well as the K<sup>d</sup>-expressing CVKD-10.6 line sensitized with the synthetic anchor peptide, and failed to recognize parent L cells (K2) or CV-1 cells treated with the same peptide. These CTL clones fail to recognize target cells treated with peptides corresponding to unrelated sequences within the A/JAP/305/57 HA molecule (Braciale, T. J., unpublished observations).

### Discussion

In this report we have examined the role of the membrane-spanning hydrophobic anchor sequence of the HA glycoprotein of influenza virus in the formation of the target antigen/MHC complex recognized by class I MHC-restricted influenza HA-specific CTL. For these studies we used a panel of H-2K<sup>d</sup>-restricted influenza HA-specific CTL clones as effectors. K<sup>d</sup>-expressing CV-1 cells infected with SV-40-based expression vectors containing different HA constructions were tested as targets for CTL recognition. The majority of the HA-specific CTL clones that were examined recognize target cells expressing a

TABLE IV  
MHC Restriction of Target Cell Sensitization by Synthetic Anchor Peptide

Exp.	CTL clone	E/T ratio	Percent specific <sup>51</sup> Cr release from target cells*			
			CVKD-10.6		CV-1	
			A/JAP/57	Peptide	A/JAP/57	Peptide
1	14-7	5:1	12	11	0.9	0.5
		20:1	33	34	0.5	0.3
	C-1	5:1	22	20	0.2	2
		20:1	41	32	0.3	2
			K7-74(K <sup>d</sup> )	K7-74(K <sup>d</sup> )	K2	K2
			A/JAP/57	Peptide	A/JAP/57	Peptide
2	11-1	5:1	39	0	0.4	0.9
		20:1	49	1	1	2
	14-7	5:1	38	47	1	1
		0:1	43	67	2	3
	C-1	5:1	47	46	0.8	3
		20:1	51	59	2	6

\* As in Table I. Target cells were either infected with A/JAP/57 virus or exposed to the 23-residue synthetic anchor peptide (final peptide concentration, 1.25 μg/ml) for the duration of the assay.

truncated HA gene that lacks the DNA sequence corresponding to the transmembrane and cytoplasmic domains of the HA molecule. Thus, expression of a gene that encodes an HA molecule that can be anchored in the lipid bilayer is not an obligate requirement for display of the HA gene product in a form recognizable by class I MHC-restricted CTL.

Although a transmembrane sequence is not required for recognition of HA by class I MHC-restricted CTL, our analyses indicate that a subpopulation of the HA-specific CTL recognize epitopes(s) located within the transmembrane domain of the molecule. Thus three CTL clones that failed to recognize either the anchor-negative HA or chimeric HA molecules that contain anchor sequences from unrelated proteins could lyse target cells sensitized by a synthetic peptide corresponding to part of the membrane-spanning anchor sequence. Sensitization by this anchor peptide was specific since only those CTL clones that did not recognize the anchor-negative HA were able to recognize peptide-treated target cells. Furthermore, synthetic peptides corresponding to other distinct regions of the HA have so far failed to sensitize histocompatible target cells for recognition by these anchor-specific CTL clones (Braciale, T. J., unpublished observations).

Two of the three anchor-specific CTL clones have previously been shown to be directed to an epitope common to most type A influenza viruses of the H2N2 subtype, while the third clone (C-1) was shown to recognize an HA epitope shared by viruses of the H1N1 and H2N2 subtypes (14). The amino acid sequences of the transmembrane domains of the A/JAP/305/57 and the A/PR/8/34 HA molecules are sufficiently homologous to account for the shared epitope defined by the C-1 clone, yet divergent enough to contain the epitopes(s) recognized by the subtype-specific clones 14-7 and A-4 (Fig. 5). Experiments are now in progress to better define these epitopes.

A critical issue raised by our observations relates to the structure of the

antigenic moieties on virus-infected cells recognized by class I MHC-restricted CTL. Early studies on CTL recognition of viral antigens favored the view that CTL target antigens needed to be displayed on the surface of virus-infected cells in their native form to render the cells susceptible to CTL-mediated lysis (22–25). More recently, Townsend et al. (8, 9) have proposed that, rather than recognizing the native viral antigens, class I MHC-restricted CTL recognize processed forms of these antigens. Their reported results concerning class I MHC-restricted CTL recognition of target cells that express the product of an HA gene lacking the sequence for the signal peptide (26), as well as our present findings on CTL recognition of the anchor-negative HA, are difficult to reconcile with recognition of the HA glycoprotein in its native form. Our identification of sequences located within the transmembrane domain as potential epitopes for CTL recognition also appears to exclude the native membrane bound form of HA as the obligatory target antigen for CTL and strongly favors the processing hypothesis.

Several groups have recently reported that class I MHC-restricted CTL (9, 10, 27, 28) or alloreactive class I MHC-specific CTL (29) can recognize synthetic peptides corresponding to portions of the viral or MHC target antigens. Our finding that a synthetic peptide corresponding to part of the transmembrane domain of HA can sensitize target cells for HA-specific CTL confirms and extends these observations to the influenza HA. In light of these results, it is attractive to consider that peptide fragments of endogeneously synthesized HA are the antigenic moieties recognized by class I MHC-restricted CTL on the surface of influenza-infected cells. The recent demonstrations of direct interactions between synthetic peptide fragments of antigens and H-2 I region products (2, 3) suggest a structural basis for restricted recognition by class II MHC-restricted T lymphocytes that may be necessary but not sufficient for T lymphocyte triggering. In view of the similarity of the T cell receptor genes used by both class I and class II MHC-restricted T lymphocytes (30), a direct interaction between a viral peptide and class I MHC products might also be a necessary step in restricted recognition by class I MHC-restricted T lymphocytes. In this regard, an important issue raised by our results concerns the mechanism by which an extremely hydrophobic peptide such as that used in these studies (Fig. 4) could directly interact with and form a stable complex with the K<sup>d</sup> gene product on the cell surface. At present, the precise nature of the antigenic moiety recognized by these anchor-specific CTL clones on the surface of influenza-infected cells is open to speculation. It is noteworthy however, that all three anchor-specific CTL clones fail to efficiently recognize the native HA polypeptide when it is introduced exogenously into the target cells as part of noninfectious influenza virions or as the isolated intact protein (4 and our unpublished observations). This is consistent with our previous observation that class I MHC-restricted CTL preferentially recognize HA moieties synthesized *de novo* in target cells rather than exogenously introduced intact HA (4).

Studies of Wabuki-Bunoti et al. (6, 31, 32) have implicated at least two other sites on the A/JAP/57 HA as potential class I MHC-restricted CTL target epitopes. These amino acid sequences were defined at the level of induction of proliferation of class I MHC-restricted CTL responses and have not as yet been

formally mapped at the level of target cell sensitization. Several distinct regions of the HA (within the HA1 subunit) have also been mapped as recognition sites of Class II MHC-restricted T lymphocytes (e.g., references 33, 34). We have examined a large panel of class II MHC-restricted HA-specific T lymphocyte clones for recognition of the synthetic anchor peptide and to date have failed to detect any anchor specific H-2I region-restricted T lymphocytes (L. Morrison, unpublished observations). Although still preliminary, this failure may reflect the inability of the anchor sequence to interact with certain Class II MHC products (2, 3, 35). Alternatively, since HA appears to be preferentially presented to class II MHC-restricted T lymphocytes by an exogenous presentation pathway (4), it is possible that the anchor sequence is not preserved during the processing of the HA glycoprotein through that putative endocytic pathway. Further analysis will be required to distinguish among these and other possibilities.

In conclusion, in this report we have demonstrated that display of *de novo* synthesized influenza HA protein as an integral constituent of the target cell plasma membrane is not required for target cell recognition by class I MHC-restricted CTL. Furthermore, amino acid residues located within the transmembrane anchor sequence of the A/JAP/57 HA appear to define antigenic epitopes for subtype-specific as well as crossreactive class I MHC-restricted CTL clones. These findings are consistent with the view that class I MHC-restricted T lymphocytes may recognize a nonintegral form of HA synthesized *de novo* and displayed by an endogeneous presentation pathway, while class II MHC-restricted T lymphocytes preferentially recognize antigens that have been modified after introduction into presenting cells via an exogenous pathway (4, 36).

### Summary

We have examined the requirement for the transmembrane hydrophobic anchor sequence of the influenza hemagglutinin (HA) in the formation of the antigenic moiety on the surface of target cells recognized by class I MHC-restricted murine CTL. For this analysis we have used a line of CV-1 monkey epithelial cells that express the transfected murine H-2K<sup>d</sup> gene product as target cells and have used recombinant SV40-based late replacement vectors to achieve expression of genes encoding wild-type and mutant forms of HA. We have found that the majority of K<sup>d</sup>-restricted HA-specific CTL clones recognize target cells that express a secreted HA molecule that lacks the transmembrane and cytoplasmic domains of the parent glycoprotein. Several K<sup>d</sup>-restricted CTL clones that recognize subtype-specific and crossreactive epitopes on HA fail to recognize the anchor-negative, secreted HA or chimeric HA molecules containing the transmembrane and cytoplasmic domains of unrelated glycoproteins. These CTL clones appear to be directed to antigenic epitopes located within the transmembrane domain of HA, as defined by their capacity to recognize target cells sensitized with a synthetic 23-amino-acid peptide corresponding to sequences within this domain. The implications of these results for class I MHC-restricted CTL recognition are discussed.

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### References

1. Zinkernagel, R. M., and P. D. Doherty. 1979. MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T cell restriction specificity, function, and responsiveness. *Adv. Immunol.* 27:52.
2. Babbitt, B., P. M. Allen, G. Matsueda, E. Haber, and E. R. Unanue. 1985. Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature (Lond.)* 317:359.
3. Buus, S., A. Sette, S. M. Conlon, D. M. Jenis, and H. Grey. 1986. Isolation and characterization of antigen-Ia complexes involved in T cell recognition. *Cell* 47:1071.
4. Morrison, L. A., A. Lukacher, V. L. Braciale, D. P. Fan, and T. J. Braciale. 1986. Differences in antigen presentation to MHC class I and class II-restricted influenza virus specific cytolytic T lymphocyte clones. *J. Exp. Med.* 163:903.
5. Guertin, D. P., and D. P. Fan. 1980. Stimulation of cytolytic T cells by isolated viral peptides and HW protein coupled to agarose beads. *Nature (Lond.)* 283:308.
6. Wabuke-Bunoti, M., D. P. Fan, and T. J. Braciale. 1981. Stimulation of anti-influenza cytolytic T lymphocytes by CNBr cleavage fragments of the viral hemagglutinin. *J. Immunol.* 127:1122.
7. Gooding, L. R., and K. A. O'Connell. 1983. Recognition by cytotoxic T lymphocytes of cells expressing fragments of the SV40 tumor antigen. *J. Immunol.* 131:2580.
8. Townsend, A. R. M., R. M. Goth, and J. Davey. 1985. Cytotoxic T cells recognize fragments of the influenza nucleoprotein. *Cell* 42:457.
9. Townsend, A. R. M., J. Rothbard, F. M. Gotch, G. Bahadur, D. Wraith, and A. J. McMichael. 1986. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell* 44:959.
10. Maryanski, J. L., P. Pala, C. Corradin, B. R. Jordan, and J. C. Cerrotini. 1986. H-2 restricted cytolytic T cells specific for HLA can recognize a synthetic HLA peptide. *Nature (Lond.)* 324:578.
11. Braciale, T. J. 1977. Immunologic recognition of influenza virus-infected cells. I. Generation of a virus strain-specific and a cross-reactive subpopulation of cytotoxic T cells in the response of type A influenza viruses of different subtypes. *Cell. Immunol.* 33:423.
12. Doyle, C., M. G. Roth, J. Sambrook, and M.-J. Gething. 1985. Mutations in the cytoplasmic domain of the influenza hemagglutinin affect different stages of intracellular transport. *J. Cell Biol.* 100:704.
13. Smith, G. L., B. R. Murphy, and B. Moss. 1983. Construction and characterization of an infectious vaccinia virus recombinant that expresses the influenza hemagglutinin gene and induces resistance to influenza virus infection in hamsters. *Proc. Natl. Acad. Sci. USA.* 80:7155.
14. Braciale, T. J., T. J. Henkel, A. E. Lukacher, and V. L. Braciale. 1986. Fine specificity and antigen receptor expression among influenza virus-specific cytolytic T lymphocyte clones. *J. Immunol.* 137:995.
15. Goodenow, R. S., I. Stroynowski, M. McMillan, M. Nicolson, K. Eakle, B. T. Sher, N. Davidson, and L. Hood. 1983. Expression of complete transplantation antigens by mammalian cells transformed with truncated class I genes. *Nature (Lond.)* 301:388.
16. Braciale, T. J., M. E. Andrew, and V. L. Braciale. 1981. Heterogeneity and specificity

- of cloned lines of influenza-virus-specific cytotoxic T lymphocytes. *J. Exp. Med.* 153:910.
17. Gething, M.-J., and J. Sambrook. 1981. Cell-surface expression of influenza hemagglutinin from a cloned DNA copy of the RNA gene. *Nature (Lond.)*. 293:620.
  18. Gething, M.-J., and J. Sambrook. 1982. Construction of influenza hemagglutinin genes that code for intracellular and secreted forms of the protein. *Nature (Lond.)*. 300:598.
  19. Roth, M., M.-J. Gething, and J. Sambrook. 1987. Intracellular transport and membrane insertion of the influenza virus glycoproteins. In *The Influenza Viruses*. R. M. Krug, editor. Plenum Publishing Corp., New York. In press.
  20. Braciale, T. J., V. L. Braciale, T. J. Henkel, J. Sambrook, and M.-J. Gething. 1984. Cytotoxic T lymphocyte recognition of the influenza hemagglutinin gene product expressed by DNA-mediated gene transfer. *J. Exp. Med.* 159:341.
  21. Andrew, M. E., A. M. Churilla, T. R. Malek, V. L. Braciale, and T. J. Braciale. 1985. Activation of virus specific CTL clones: antigen-dependent regulation of interleukin 2 receptor expression. *J. Immunol.* 134:920.
  22. Sugamura, K., R. Shemizu, J. Zarling, and F. H. Bach. 1977. Role of Sendai virus fusion-glycoprotein in target cell susceptibility to cytotoxic T cells. *Nature (Lond.)*. 270:25.
  23. Gething, M.-J., V. Koszinowski, and M. Waterfield. 1978. Fusion of Sendai virus with the target membrane is required for T cell cytotoxicity. *Nature (Lond.)*. 275:689.
  24. Braciale, T. J., and K. L. Yap. 1978. Role of viral infectivity in the induction of influenza virus-specific cytotoxic T cells. *J. Exp. Med.* 147:1236.
  25. Kurrle, R., H. Wagner, M. Rollinghoff, and R. Rott. 1979. Influenza virus-specific T cell-mediated cytotoxicity: integration of the virus antigen into the target cell membrane is essential for target cell formation. *Eur. J. Immunol.* 9:107.
  26. Townsend, A., J. Bastin, K. Gould, and G. Brownlee. 1986. Cytotoxic T lymphocytes recognize influenza hemagglutinin that lacks a signal sequence. *Nature (Lond.)*. 324:575.
  27. McMichael, A., F. Gotch, and J. Rothbard. 1986. HLA B37 determines an influenza A virus nucleoprotein epitope recognized by cytotoxic T lymphocytes. *J. Exp. Med.* 164:1397.
  28. Parham, P., C. Clayberger, S. Zorn, D. Ludwig, G. Schoolnik, and A. M. Krensky. 1987. Inhibition of alloreactive cytotoxic T lymphocytes by peptides from the  $\alpha 2$  domain of HLA-A2. *Nature (Lond.)*. 325:625.
  29. Gotch, F., J. Rothbard, K. Howland, A. Townsend, and A. McMichael. 1987. Cytotoxic T lymphocytes recognize a fragment of influenza virus matrix protein in association with HLA-A2. *Nature (Lond.)*. 326:881.
  30. Kronenberg, M., G. Sui, L. E. Hood, and N. Shastuk. 1986. The molecular genetics of the T cell antigen receptor and T cell antigen recognition. *Annu. Rev. Immunol.* 4:529.
  31. Wabuke-Bunoti, M. A. N., and D. P. Fan. 1983. Isolation and characterization of a CNBr cleavage fragment of influenza viral hemagglutinin stimulatory for mouse cytolytic T lymphocytes. *J. Immunol.* 130:2386.
  32. Wabuke-Bunoti, M., A. Taku, D. Fan, S. Kent, and R. Webster. 1984. Cytolytic T lymphocyte and antibody responses to synthetic peptides of influenza virus hemagglutinin. *J. Immunol.* 133:2194.
  33. Hackett, C. J., B. Dietzschold, W. Gerhard, B. Ghrist, R. Knorr, D. Gillessen, and F. Melchers. 1983. 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. *J. Exp. Med.* 158:294.

34. Lamb, J. R., D. Eckels, P. Lake, J. N. Woody, and N. Green. 1982. Human T-cell clones recognize chemically synthesized peptides of influenza hemagglutinin. *Nature (Lond.)*. 300:66.
35. Guillet, J.-G., M.-Z. Lai, T. Briner, S. Buus, A. Sette, H. Grey, J. A. Smith, and M. Gefer. 1987. Immunological self, nonself discrimination. *Science (Wash. DC)*. 235:865.
36. Braciale, T. J., L. A. Morrison, M. T. Sweetser, J. Sambrook, M.-J. Gething, and V. L. Braciale. 1987. Antigen presentation to class I and class II MHC-restricted T lymphocytes. *Immunol. Rev.* In press.