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CTL Escape Viral Variants

I. Generation and Molecular Characterization

HANNA LEWICKI,* ANTOINETTE TISHON,* PERSEPHONE BORROW,* CLAIRE F. EVANS,* JEAN E. GAIRIN,† KLAUS M. HAHN,* DAVID A. JEWELL,‡ IAN A. WILSON,‡ and MICHAEL B. A. OLDSTONE*,[1]

*Division of Virology, Department of Neuropharmacology, The Scripps Research Institute, La Jolla, California 92037; †Laboratoire de Pharmacologie et de Toxicologie Fondamentales, CNRS, 31077 Toulouse Cedex, France; and ‡Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037

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Cytotoxic T lymphocytes (CTL) play a pivotal role in preventing persistent viral infections and aborting acute infections. H-2D^b-restricted CTL optimally recognize a specific peptide of 9 to 11 amino acids (aa) derived from a viral protein and held in place (restricted) by a MHC class I glycoprotein on the surfaces of infected cells. Only three peptide sequences with the appropriate D^b motif from lymphocytic choriomeningitis virus Armstrong strain (LCMV) are known to be presented to CTL by H-2D^b molecules; they are from the glycoproteins (GP), residues 33–41 KAVYNFATC (GP1) and 276–286 SGVENPGGYCL (GP2), and the nucleoprotein (NP), 396–404 FQPQNGQFI. Incubation of virally infected H-2^b cells with CTL clones that recognize only GP1, GP2, or NP leads to the selection of viral variants which upon infecting cells bearing H-2^b molecules, escape recognition by CTL of the appropriate specificity. Nucleic acid sequencing showed a single mutation in GP1 (aa 38 F→L), GP2 (aa 282 G→D), or NP (aa 403 F→L) in the variant viruses. When wild-type (wt) LCMV peptides and the three variant peptides (GP1, GP2, NP) were synthesized and subjected to a competitive inhibition binding assay, no differences in binding affinity for H-2D^b were found between the wt and variant peptides. Uninfected cells coated with the wt peptide were recognized and lysed by the appropriate CTL clone or by *in vivo*-primed bulk CTL, but similar targets coated with the GP1, GP2, or NP variant peptides were not. This result, coupled with computer graphic analysis of these variant peptides with the recently solved three-dimensional structure for the D^b MHC class I molecule, placed the side chain of the mutated residues on the outer surface of the MHC-peptide complex and accessible to the T cell receptor. Ala substitution at GP residue 38 or 282 or at NP 403 also abrogated CTL recognition and lysis. Inoculation of any one of the mutated viral variants into mice produced an effective CTL response to the other two nonmutated GP or NP peptides, suggesting that production of biologically relevant CTL escape virus variants *in vivo* requires selection of mutations in more than one and likely all the CTL epitopes, a low probability event.

INTRODUCTION

The immune response specific for viral antigens can be segregated into two distinct pathways: humoral (antibody) and cellular (T lymphocyte). Antibodies generally recognize antigens circulating freely in the blood and other fluids, whereas T lymphocytes interact with antigens in the form of processed peptides bound to host cells and presented in a groove between the two α helices of the major histocompatibility (MHC) glycoprotein (Zinkernagel *et al.*, 1974; Townsend *et al.*, 1986; Bjorkman *et al.*, 1987; Oldstone, 1994; Whitton and Oldstone, 1995). Cytotoxic T lymphocytes (CTL) most often bear the CD8 molecule and recognize and interact with virally infected cells expressing viral antigen (peptide) presented by class I MHC molecules.

Class I MHC-restricted CTL play a pivotal role in aborting and preventing acute (Oldstone, 1994; Whitton and Oldstone, 1995; Byrne and Oldstone, 1984; Lin and Askonas, 1981; Zinkernagel and Welsh, 1976; Klavinskis *et al.*, 1990; Oldstone *et al.*, 1992; Whitton *et al.*, 1993) and persisting viral infections (Whitton *et al.*, 1993; Oldstone *et al.*, 1993, 1986; Ahmed *et al.*, 1987; Tishon *et al.*, 1993). MHC class I presentation utilizes primarily a cytosolic pathway (Whitton and Oldstone, 1995, 1989; Braciale, 1992; Braciale and Braciale, 1992) and MHC class I molecules are found on nearly all nucleated cells in the body, except neurons (Joly *et al.*, 1991; Lampson, 1987). The MHC-bound peptide sequence is linear, resulting from proteolytic fragmentation of viral protein synthesized within the cell (Townsend *et al.*, 1986; Whitton and Oldstone, 1995, 1989; Braciale, 1992; Braciale and Braciale, 1992). The optimal length of such peptides is 8 to 11 amino acids (aa) (van Bleek and Nathanson, 1990; Falk *et al.*, 1991; Young *et al.*, 1994; Oldstone, 1994; Fremont *et al.*, 1992; Matsumura *et al.*, 1992; Gairin *et al.*, 1995; Garboczi *et al.*, 1994; Madden *et al.*, 1993).

Viruses have evolved a variety of mechanisms to escape the effects of CTL (reviewed by Oldstone, 1991; Koup, 1994). By definition, then, a persisting virus is one

[1] To whom reprint requests should be addressed.

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that has successfully solved the puzzle of escaping CTL immune surveillance. One such escape strategy is for a virus to alter (mutate) amino acids within the sequence that binds to MHC in a manner that prevents CTL recognition.

To analyze this particular strategy, we utilized cells transfected with the MHC class I D^b molecule, as well as recombinant technology and peptides from lymphocytic choriomeningitis virus (LCMV) to determine the optimal amino acid sequence(s) bound by D^b molecules and recognized by LCMV-specific D^b-restricted CTL. It was found that only three peptides served as CTL epitopes in H-2^b mice: glycoprotein residues 33–41 KAVYNFATC (GP1) and 276–286 SGVENPGGYCL (GP2) and nucleoprotein residues 396–404 FQPQNGQFI (NP) (Gairin *et al.*, 1995; Whitton *et al.*, 1988; Oldstone *et al.*, 1988, 1991; Yanagi *et al.*, 1992). We then determined whether CTL escape viral variants with mutations in these epitopes were generated when virus was grown in the presence of antiviral CTL clones. Here we demonstrate that: (1) viral mutants escaping recognition by CTL can be generated by single aa mutations in GP1, GP2, or NP, the three D^b-restricted CTL epitopes; (2) the mutated peptide, in all instances, bound as well as the authentic (wild type) viral peptide to the MHC molecule but failed to sensitize MHC-compatible target cells for CTL lysis; and (3) virus mutated in any one CTL epitope still generated CTL immune responses to the other two epitopes. The implications of these findings as regards the probability that biologically relevant CTL escape variant viruses will emerge *in vivo* are discussed.

MATERIALS AND METHODS

Mice

Mouse strains C57BL/6 (H-2^{bb}) and BALB/cByJ (H-2^{dd}) were obtained from the breeding colony at The Scripps Research Institute. Mice used were 6 to 12 weeks of age.

Cell lines, viruses, and CTL clones

The MC57 (H-2^b), BALB CI7 (H-2^d), RMA (H-2^b) and its variant RMA.S, and T2-D^b cell lines were maintained in continuous culture in the laboratory and routinely checked to ensure they were mycoplasma-free, as described (Whitton and Oldstone, 1989; Gairin *et al.*, 1995; Whitton *et al.*, 1988). All lines were maintained in MEM or RPMI medium supplemented with 7% fetal calf serum, L-glutamine, and penicillin–streptomycin. Viruses used were LCMV (Armstrong strain) clone 53b and recombinant vaccinia viruses (VV) that expressed LCMV GP, NP, GP1, or GP2 (Whitton *et al.*, 1988, 1989; Oldstone *et al.*, 1988, 1991).

CTL clones were the following: LCMV GP1 (VI-45-7) recognizing GP aa 33–41, LCMV GP2 (77-82 or 228) recognizing GP aa 276–286, and LCMV NP (NP18) recognizing NP aa 396–404, all H-2^b (D^b)-restricted, as well as LCMV NP clone (HD8) that recognized NP aa 118–127 and was H-2^d (L^d)-restricted. Data concerning the generation, characterization, and culturing conditions for these clones have been published (Whitton *et al.*, 1988, 1989; Oldstone *et al.*, 1988, 1991; Yanagi *et al.*, 1992). The specificity of each clone's reactivity against LCMV-infected targets is shown in Table 1.

Selection of viral variants

The procedure followed is illustrated in Fig. 1 and was modified from the report of Aebischer *et al.* (1991). Briefly, MC57 cells in the log growth phase were incubated with virus for 1 hr at a low multiplicity of infection (m.o.i. 0.001). After repeated washes, infected cells were mixed with either GP1, GP2, or NP-specific CTL clones at a ratio of 1:50 (LCMV-infected cells:CTL clone). Supernatant was collected 48 hr later; viral plaques were then picked, cloned three times, and used as master seeds to prepare viral stocks in BHK cells. Resultant viruses were tested for the ability to infect either MC57 or BALB CI7 cells and render them targets for CTL recognition and lysis or for the ability to generate primary CTL *in vivo*. Virus selected by GP1 clone was called GP1V, by GP2 clone GP2V, and by NP clone NPV.

CTL generation and detection

Primary LCMV-specific CTL were generated by inoculating mice intraperitoneally with 1×10^5 PFU of LCMV ARM (wild type, wt) or 1×10^6 PFU of the various CTL escape variants. Spleens were obtained 7 days later, and single-cell suspensions of splenic lymphocytes (free of erythrocytes) were placed in RPMI 1640 media and tested for cytotoxicity in a 5- to 6-hr ^{51}Cr assay *in vitro* as described (Whitton *et al.*, 1988; Oldstone *et al.*, 1988).

To generate target cells for these assays, H-2^b or H-2^d cell lines were infected 48 hr prior to use with LCMV wt or variants at an m.o.i. of 1 or 18 hr prior to use with VV/LCMV recombinants at an m.o.i. of 3. Various synthetic peptides were incubated with uninfected H-2^b or H-2^d targets at concentrations of 10^{-5} to 10^{-14} *M* and then the mixture was reacted with CTL clones or CTL generated after a primary immunization as described (Gairin *et al.*, 1995; Whitton *et al.*, 1988, 1989; Oldstone *et al.*, 1988, 1991; Yanagi *et al.*, 1992). Supernatant was harvested, and specific ^{51}Cr release was calculated by the following formula: [(sample release − spontaneous release)]/[(total release − spontaneous release)] × 100.

Nucleic acid sequence analysis of CTL escape variants

Sequences of CTL escape viral variants were determined by direct RNA sequencing. RNA was prepared from virus-infected BHK or MC57 cells at 48 or 72 hr

TABLE 1

Specificity of CTL Clones Used to Generate CTL Escape Viral Variants

		% Specific ^{51}Cr released from targets					
		H-2^b					H-2^d
			VV Recombinant				
Effector CTL	E:T ratio	LCMV ARM	NP	GP	GP1	GP2	LCMV ARM
Clones D^b-restricted							
GP1 VVI-45	5:1	**51**	1	**46**	**44**	0	1
GP2 77-82-14	5:1	**62**	0	**58**	1	**49**	0
NP18	5:1	**39**	**29**	1	0	1	1
Clones L^d-restricted							
NP HD8	5:1	0	0	0	0	1	**68**
Primary Day 7 Spleen							
C57BL/6 H-2^b	50:1	**49**	**22**	**47**	**21**	**19**	2
BALB H-2^d	50:1	0	0	5	0	4	**46**

Note. CTL clones were used at effector to target (E:T) ratios of 5:1 and 2.5:1 with data for 5:1 shown. Primary (bulk) CTL were generated by inoculating C57BL/6 or BALB/cByJ mice ip with 1×10^5 PFU of LCMV and harvesting lymphocytes from their spleens 7 days later. The cytotoxicity assay was carried out as described under Materials and Methods. Numbers represent the mean of triplicate samples and variance among samples was <10%. Values in bold represent significant values.

following infection at an m.o.i. of 0.1–0.5 PFU/cell. Cells were lysed in a Tris-buffered 4 *M* guanidine–isothiocyanate solution. DNA was sheared by passing the lysate three times through a 21-gauge needle. RNA was isolated by adding 0.1 volume of 2 *M* sodium acetate, pH 4.0, and 0.2 volume of chloroform:isoamyl alcohol (49:1), extracting with 1 volume of acid phenol, and precipitating with isopropanol. The RNA was resuspended in TE (10 m*M* Tris, 1 m*M* EDTA), precipitated with ethanol, resuspended in TE at 5–10 mg/ml, and stored at −20°. Sequence analysis was performed as previously described (Salvato *et al.*, 1991). Briefly, ^{32}P-labeled LCMV S RNA-specific oligonucleotide primers and dideoxynucleotides were used to perform primer extension sequencing catalyzed by reverse transcriptase. The primers used were as follows: to sequence the region containing the H-2^b CTL epitope in GP1 5′-AGAAGTAGGAAACTGATC-AATGC-3′, in GP2 5′-CAACTGCTGTGTTCCCGAAACAC-3′, and in NP 5′-CGCTCCTACATGGATTGAC-3′. Approximately 50 μg of total infected-cell RNA was used for each sequencing reaction.

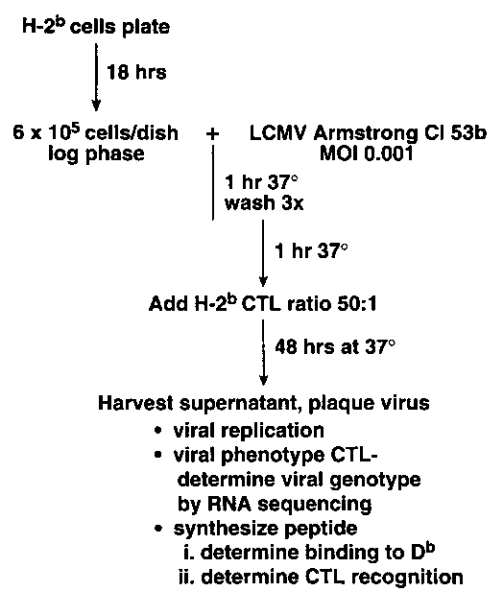


FIG. 1. Schematic outline of the procedure followed to generate and characterize CTL escape variants.

Peptide synthesis, peptide labeling, and MHC binding assay

Peptides were synthesized using the solid-phase method of Merrifield (1963) with an automated peptide synthesizer (Model 430A; Applied Biosystems, Inc., Foster City, CA). Peptides were cleaved from their insoluble polystyrene resin beads by hydrogen fluoride, extracted, lyophilized, and purified by HPLC. Purity of peptides used was >98%. Identity of purified peptides was confirmed by fast atom bombardment mass spectrum analysis. The GP2 peptide SGVENPGGYCL was labeled by the chloramine T method. Briefly, 10 μl of peptide (2.5 mg/ml) was added to a 1.5-ml conical test tube containing 50 μl of phosphate buffer (0.25 *M*, pH 7), 1 mCi ^{125}I, and 10 μl of chloramine T (4.2 mg/ml). After 1 min, 20 μl of sodium metabisulfite (4.2 mg/ml) was added. One minute later, 20 μl of potassium iodide was added, followed by 400 μl of PBS containing 1% BSA and 0.05% Tween 20. After the mixture was placed on a Sephadex G-25M column, fractions containing ^{125}I-peptide were collected.

TABLE 2

Generation of LCMV Escape Variants to GP1, GP2, and NP CTL Epitopes Restricted by H-2^b (D^b) MHC Class I Molecule

		^{51}Cr released from H-2^b target (MC57) infected with								^{51}Cr released from H-2^d target (BALB C17) infected with		
						VV/LCMV					VV/LCMV	
CTL used	E:T	LCMV wt	GP1V	GP2V	NPV	NP	GP	GP1	GP2	LCMV wt	GP	NP
Day 7 primary spleen CTL												
H-2^b												
LCMV wt	50:1	55	50	46	28	18	24	46	40	2	ND	ND
	25:1	38	30	33	18	10	19	35	24	1	ND	ND
GP1V	50:1	60	65	58	38	42	39	4	44	5	ND	ND
	25:1	52	40	54	28	34	31	1	32	2	ND	ND
GP2V	50:1	65	38	52	30	50	28	55	1	3	ND	ND
	25:1	49	24	46	21	37	18	34	4	2	ND	ND
NPV	50:1	59	54	34	38	3	34	40	21	3	ND	ND
	25:1	50	40	24	28	1	21	38	14	1	ND	ND
H-2^d												
LCMV wt	50:1	1	ND	ND	ND	1	0	ND	ND	68	2	56
NPV	50:1	0	ND	ND	1	2	1	ND	ND	64	1	71
CTL clones												
H-2^b												
GP1 VVI-45	5:1	32	1	25	18	0	19	35	0	0	ND	ND
GP2 228	5:1	52	54	0	48	0	26	0	22	0	ND	ND
GP2 77-82	5:1	68	55	1	47	0	58	0	69	0	ND	ND
NP18	5:1	62	51	59	0	55	1	2	3	0	ND	2
H-2^d												
NP HD8	5:1	1	ND	ND	2	1	1	ND	ND	61	1	52

Note. GP1V, GP2V, and NPV were selected as described under Materials and Methods and outlined in Fig. 1. Day 7 primary splenic CTL were obtained from H-2^b C57BL/6 or H-2^d BALB/cByJ mice. The 5- to 6-h ^{51}Cr release assay is described under Materials and Methods. Numbers shown represent the mean of triplicate samples. The variance was <10%, and significant elevations in ^{51}Cr release are shown in bold. Results were repeated in two other experiments. E:T, effector to target ratio. ND, not determined.

The binding assay used RMA.S or T2-D^b cells as described (Gairin *et al.*, 1995; Gairin and Oldstone, 1993). Briefly, 200 μl of cells (10×10^6 cells/ml) and 50 μl of ^{125}I-peptide (5×10^6 cpm) were incubated for 1–2 hr at 37° in the presence of 27.5 μl of unlabeled competitor peptide (10^{-4} to 10^{-7} *M*, final concentration) or medium alone. Cells were washed three times with ice cold medium or 1% BSA–PBS and the radioactivity bound to cells was counted in a gamma counter. Percentage of inhibition of binding of the labeled peptide by the competitor peptide was calculated by the following formula: [cpm (medium alone) − cpm (competitor)]/[cpm (medium alone)] × 100.

Computer modeling

The LCMV peptides GP1 (33–41, KAVYNFATC) and NP (396–404, FQPQNGQFI) were modeled into the binding site of the X-ray structure of H-2D^b (Young *et al.*, 1994) by replacing those side chains differing from the influenza nucleoprotein peptide 366–374, ASNENMETM (PDB Accession ID Code 1hoc). Of the amino acid replacements made to the crystal structure only the M to F (38) in the GP1 caused a steric clash. The best amino acid rotomers were manually searched for with the computer program O (Jones *et al.*, 1991). The approximate conformations of the bound peptides were decided upon using this manual method and the final conformation was determined using 100 cycles of energy minimization in the cvff force field of DISCOVER (Biosym, San Diego, CA). The manually fit GP1 and NP peptide conformations have root mean square deviations (rmsd) for the mainchain atoms of 0.58 and 0.25 Å, relative to the influenza peptide, and for the final energy-minimized models of 0.59 and 0.29 Å, respectively. The geometry of the modeled complexes was analyzed using PROCHECK (Laskowski *et al.*, 1993) and found to conform to better than the statistical average of PDB-deposited structures. No further analysis of the modeled complexes was deemed appropriate.

RESULTS

Generation of CTL escape variants for GP1, GP2, and NP epitopes of LCMV

Viral variants capable of escaping CTL killing were developed by following the protocol displayed in Fig. 1

and using selective pressure exerted by cloned CTL specific for LCMV ARM GP1, GP2, or NP epitopes, respectively. As seen in Table 2, escape variants generated to GP1 (GP1V), when infecting syngeneic H-2^b targets, were not killed by GP1 CTL clone VV1-45 but were killed by CTL clones to GP2 and NP. Similarly, GP2V-infected H-2^b targets were not lysed by GP2 CTL clones but were killed by GP1 and NP H-2^b-restricted CTL clones, and H-2^b cells infected by NPV were not lysed by H-2^b NP CTL clones but were killed by GP1 and GP2 CTL clones. VV recombinants expressing GP1, GP2, or NP produced the results shown in Table 2, and the outcomes were similar when uninfected MC57 H-2^b targets were coated with peptides for either GP1 (aa 33–41), GP2 (aa 276–286), or NP (aa 396–404).

Table 2 also indicates that inoculation of GP1V into C57BL/6 mice failed to generate primary CTL to GP1 epitopes but did generate CTL to GP2 and NP epitopes, whereas inoculation of GP2V failed to elicit CTL to GP2 but did elicit CTL to GP1 and NP. Neither GP1V or GP2V elicited a CTL response to LCMV GP following inoculation into H-2^d BALB mice, as expected since such mice fail to make a CTL response to LCMV ARM wt GP (Whitton *et al.*, 1993, 1988, 1989; Oldstone *et al.*, 1993, 1988, 1991; Yanagi *et al.*, 1992). NPV generated CTL responses to GP1 and GP2 expressed on H-2^b targets and NP expressed on H-2^d targets, but failed to generate a NP CTL response for H-2^b targets. The NP CTL epitope restricted by H-2^d (L^d) targets is located at the amino end of the NP (aa 118–127), whereas the epitope restricted by H-2^b (D^b) is located at the carboxy end at aa 396–404.

Lastly, as anticipated from the data in Table 2, intracerebral inoculation of 10 PFU of GP1V, GP2V, or NPV into C57BL/6 or BALB mice led to CTL-mediated acute CNS death 6–9 days postinoculation with kinetics similar to that observed with LCMV wt (data not shown).

Biochemical abnormality (mutation) of CTL escape variants GP1V, GP2V, and NPV

Next we determined the RNA sequence changes that occurred in the CTL escape variants. Biological phenotypes shown in Table 2 remained intact when each cloned variant was passed on BHK cells to make a sufficient quantity of viral RNA for sequencing. Sequence analysis of GP1V showed a single point mutation of F to L at aa 38 in the GP1 CTL epitope, GP2V had a point mutation of G to D at aa 282, and NPV had an F to L mutation at aa 403. These data are illustrated in Fig. 2.

Synthesized peptides from GP1V, GP2V, and NPV CTL epitopes bind as well to MHC class I D^b molecules as do wt GP1, GP2, and NP peptides but fail to sensitize targets for CTL-mediated lysis

Next we synthesized CTL epitope peptides for wt GP1, GP2, and NP as well as for GP1V, GP2V, and NPV and measured their ability to bind to H-$2D^b$. Initial experiments measured the upregulation of MHC expression on RMA.S cells by these peptides using the mouse monoclonal antibody 28-14-8s anti-α3 H-$2D^b$ and a fluorescinated antibody to mouse IgG (Joly *et al.*, 1991; Gairin *et al.*, 1995; Gairin and Oldstone, 1993). Negative controls were medium alone or an irrelevant mouse monoclonal antibody. RMA.S cells grown at 25° expressed enhanced amounts of empty H-$2D^b$ molecules on their surfaces (Gairin and Oldstone, 1993; Ljunggren *et al.*, 1990). When the cells were incubated at 37° in the presence or absence of increasing concentrations of various peptides, the amount of stabilized H-$2D^b$ could be measured by FACS. According to this assay GP1V, GP2V, and NPV stabilized the expression of D^b equivalently to GP1, GP2, and NP wt peptides (data not shown).

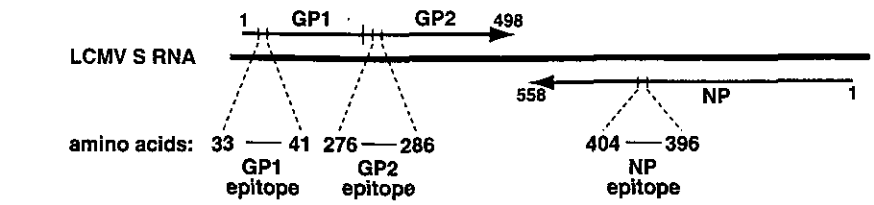
To quantitate peptide binding, we used ^{125}I to label the tyrosine at position 284 of the wt virus GP2 epitope peptide (GP2 CTL epitope, aa 276–286, SGVENPGGYCL) or the mutant peptide from GP2V (SGVENPDGYCL) and showed that both peptides bound to D^b molecules on RMA.S and T2-D^b cells. We then tested competitive binding of the wt GP2 radiolabeled peptide and either unlabeled wt GP2 or GP2V peptides over a molar range of 10^{-4} to 10^{-7}. As shown in Fig. 3a, the binding to D^b was equivalent in both situations. However, coating of MC57 H-2^b target cells with GP2V peptide did not lead to CTL lysis of the target cells by either GP2 CTL clones or primary Day 7 CTL at a peptide concentration of 10^{-6} *M* (Fig. 3a). By contrast, coating target cells with wt GP2 peptide led to CTL lysis at 10^{-11} *M*.

Next, GP1V and wt GP1 peptide, and NPV and wt NP peptide, were similarly studied for their ability to compete against ^{125}I wt GP2 peptide. As seen in Fig. 3b for GP1V and wt GP1 peptides and in Fig. 3c for NPV and wt NP peptides, coating by neither of the peptides from CTL escape variants caused CTL lysis of target cells, but both variant peptides bound to D^b molecules as well as did peptides from wt LCMV ARM. These results were repeated in several experiments.

Next we determined the replication kinetics of GP1V, GP2V, NPV, and LCMV ARM wt in MC57 and BHK cells. Yields of virus in tissue fluids and numbers of inoculated cells containing viral antigens (Fig. 4) were similar during the 72-hr observation period of infection.

Computer graphic analysis of CTL escape variant peptides in relationship to the D^b molecule

Peptides that bind to the D^b MHC class I molecule have a motif in which an Asn (N) at position 5 and hydrophobic aa or Met (M) at the carboxy terminus serve to anchor the peptide in the MHC class I binding groove (Young *et al.*, 1994; Matsumura *et al.*, 1992; Gairin *et al.*, 1995; Falk *et al.*, 1991). We compared the structure of the 9-mer LCMV GP1 and NP CTL epitopes with the



Virus	Amino Acid Sequence (N --> C) of: GP1 epitope	GP2 epitope	NP epitope
Wt LCMV Armstrong	33 K A V Y N F A T C 41	276 S G V E N P G G Y C L 286	396 F Q P Q N G Q F I 404
GP1V	K A V Y N [L] A T C (codon change UUU → UUG)	as Wt	as Wt
GP2V	as Wt	S G V E N P [D] G Y C L (codon change GGU → GAU)	as Wt
NPV	as Wt	as Wt	F Q P Q N G Q [L] I (codon change UUC → CUC)

FIG. 2. Diagram to illustrate the nucleotide and resulting amino acid changes found in CTL escape variant viruses GP1V, GP2V, and NPV.

known structure of an influenza virus NP D^b-restricted peptide (ASNENMETM, aa 366–374) using the D^b coordinates (Young *et al.*, 1994) (Fig. 5). The modeled structures of these peptides with the mutated residues, F for aa 38 of GP1 and F for aa 403 of NP, show that both residues extend out and away from the MHC molecule and thus occupy a position in the model where they are available for recognition by the T cell receptor (TCR). In addition, both F38 and F403 may occupy lateral shallow pockets on the top surface of the MHC binding groove. Rebuilding of the GP1 F38 mutant into the D^b influenza peptide structure required alteration of the mainchain ψ angle of residue 38 to fit the pocket observed to be occupied by M371 of the influenza peptide (Fig. 5).

Ala substitution for the peptide residue 6 or 8 outside the MHC-D^b peptide-binding groove can abrogate recognition of LCMV GP1 and NP peptides to cytotoxic T lymphocytes

Further analysis of the models described above and illustrated in Fig. 5 suggested that the third, fifth, and ninth amino acid residues of GP1 and NP help to anchor the peptides in the D^b peptide binding groove, while side chains in the sixth position for GP1 and the eighth position for NP would ordinarily be free to interact with the TCR. To experimentally test this possible interaction, Ala substitutions were made for the GP1 and NP peptides at positions 3, 5, 6, 8, and 9. As shown in Table 3 for GP1 peptide, Ala substitution for Phe at residue 6 or for Thr at residue 8 abrogates CTL lysis while a corresponding Ala substitution for Val at residue 3 or for Cyt at residue 9 did not abrogate CTL-mediated lysis, in agreement with the computer graphic model.

A similar study of the D^b-restricted NP peptide (FQPQNGQFI) indicated that Ala substitutions at the third, sixth, and eighth positions aborted CTL-mediated lysis while Ala substitutions at the ninth residue did not (Table 3). As expected and shown earlier (Gairin and Oldstone, 1993), substitutions at the fifth position of the D^b-restricted LCMV peptides that changed the anchoring Asn residue aborted CTL recognition. For the GP2 peptide, Ala substitution for G at position 6 abrogated CTL lysis. By contrast, Ala substitution of the authentic residue at positions 8, 9, 10, or 11 of the GP2 peptide did not abort CTL-mediated killing (data not shown).

DISCUSSION

This report evaluates the ability of a virus to escape CTL recognition while undergoing the selective pressures of CTL reactivity toward viral peptide(s) bound within the groove of MHC class I. Our results clearly indicate that, under the selective pressure of CTL clones, CTL escape variant viruses can be generated *in vitro* for all three known LCM viral peptides that are bound to

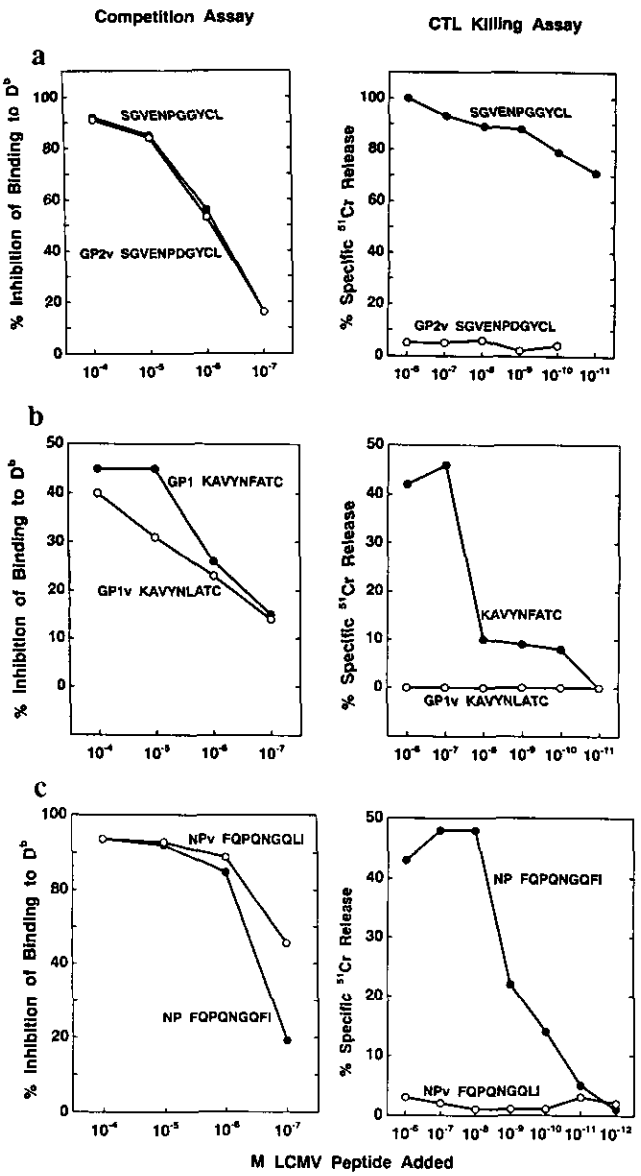


FIG. 3. Wt and CTL escape mutant epitopic peptides bind equivalently to H-2D^b MHC class I molecules, but the mutant peptides are unable to sensitize H-2^b target cells to lysis by epitope-specific CTL clones. On the left are shown competitive binding assays in which the abilities of (a) GP2 wt and GP2V peptides, (b) GP1 wt and GP1V peptides, and (c) NP wt and NPV peptides to compete with ^{125}I-labeled GP2 wt peptide for binding to H-2D^b MHC class I molecules on RMA.S cells were compared. On the right are shown ^{51}Cr release assays for CTL activity, in which the abilities of (a) GP2 wt and GP2V peptides to sensitize H-2^b target cells to lysis by CTL clone 77-82, (b) GP1 wt and GP1V peptides to sensitize H-2^b target cells to lysis by CTL clone VV1-45, and (c) NP wt and NPV peptides to sensitize H-2^b target cells to lysis by CTL clone NP18, were investigated. See Materials and Methods for details of both assays.

and presented by D^b MHC class I molecules. Because the selection occurs by way of a TCR-dependent recognition process, the mutated residues GP1 aa 38 F→L, GP2 aa 282 G→D, and NP aa 403 F→L would be most expected to lie in positions that point out and away from the MHC groove and have the ability to interact directly with the TCR. Indeed, molecular modeling of the two 9-mer peptides GP1 and NP, as shown in Fig. 5, suggests that this is the case. In only two other instances have multiple peptide structures been determined for different single peptides bound to a class I molecule. In H-2K^b, peptides of different lengths (8- and 9-mers) are accommodated once fixed in the groove by a bulging out of the central region of the peptide (Fremont *et al.*, 1992). For HLA-A2, five different peptide complexes have shown that different sequences can also fit by "zig-zagging" along the center of the groove and make new or additional lateral (i.e., side-to-side) contacts with the groove rather than simply bulging out of the groove (Madden *et al.*, 1993). Interestingly, the Ala substitutions at positions in the GP1, GP2, and NP peptides that gave rise to CTL escape mutants, GP positions 38 (F→L) and 282 (G→D) and NP position 403 (F→L), also caused abrogation of CTL recognition of these peptides when presented by D^b.

The GP2 and GP2V were not modeled because their optimal size is an 11-mer (Gairin *et al.*, 1995), and the addition of two residues would result in a substantially more speculative computer model. When Aebischer *et al.* (1991) generated LCMV WE mutants which escaped recognition by CTL clones specific for the GP2 epitope of LCMV WE, aa 275–289, they found that the most common mutation producing CTL escape was at the D^b-specific anchor residue N at position 5 (aa 280) (Falk *et al.*, 1991), although CTL escape could also be achieved by a G to D mutation at aa 282. In contrast to their results and in keeping with ours, Lill *et al.* (1992), who isolated and analyzed CTL escape variants of simian virus 40 large tumor antigen, a viral protein which contains four H-2D^b-restricted CTL epitopes, found that the point mutations responsible for CTL escape in their system all produced changes in amino acids important for recognition by the T cell receptor.

The LCMV ARM CTL response in H-2^b mice was precisely mapped to two GP (aa 33–41, aa 276–286) and one NP (aa 396–404) peptide sequences (Gairin *et al.*, 1995; Whitton *et al.*, 1988; Oldstone *et al.*, 1988, 1991; Yanagi *et al.*, 1992). We have shown that point mutations arising in any of these three peptide sequences can be selected as a result of CTL pressure. A corresponding scenario of escape variants, but directed to different peptide residues, emerged after using neutralizing monoclonal antibodies to LCMV ARM (Buchmeier *et al.*, 1981) as well as to other viruses (Laver *et al.*, 1979; Laver and Webster, 1973; Yewdell *et al.*, 1979; Wiktor and Koprowski, 1978; Narayan *et al.*, 1977). When the total number of LCMV ARM GP (558) and NP (498) aa residues are considered, the probability that a single mutation will occur in any of the known H-2^b CTL epitopes for LCMV is 0.0082 (27 bases GP1 + 33 bases GP2 + 27 bases NP/10,600 bases), assuming that the point mutations occur randomly. However, even a mutation significant enough

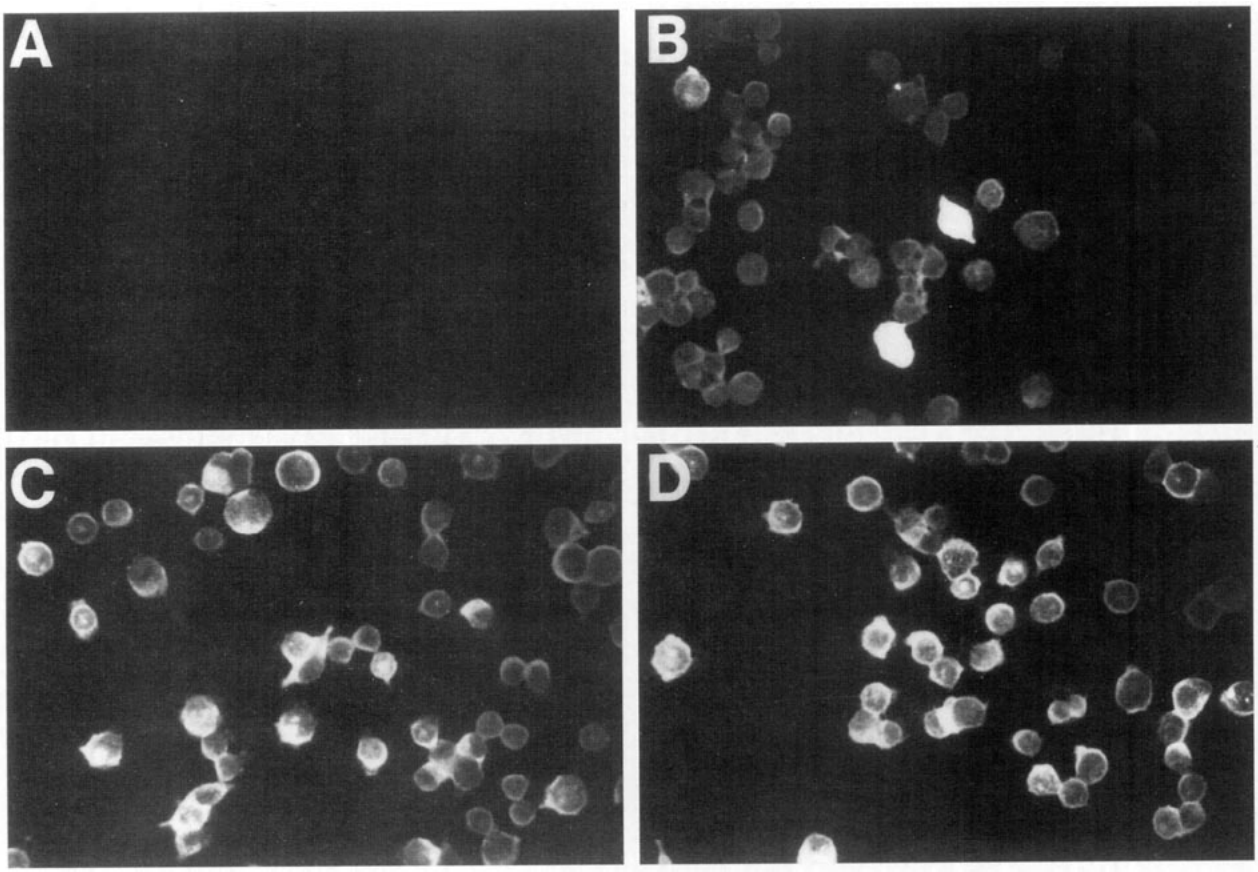


FIG. 4. GP1V, GP2V, and NPV infected and replicated in MC57 target cells. (A) Expression of LCMV NP in mock-infected MC57 cells, (B) expression in GP1V-infected cells, (C) expression in GP2V-infected cells, and (D) expression in NPV-infected cells 48 hr after challenge with these various viruses at an m.o.i. of 1. Similar results were seen with an m.o.i. of 0.1 or of 3. Viral antigen was detected by using monoclonal mouse antibody LCMV NP 113 to LCMV NP and a fluoresceinated goat anti-mouse IgG.

to allow generation of a CTL escape variant to any one of the three CTL epitopes still leaves an efficient CTL response *in vivo* for the two remaining epitopes (Table 2). Whether generation of escape mutations in two of the three known H-2^b CTL epitopes of LCMV is sufficient to produce a biologically relevant escape phenotype is currently unclear. Recently published results of Moskophidis and Zinkernagel (1995) show that an LCMV WE virus with mutations in both the GP1 and GP2 CTL epitopes was eliminated much more slowly than the wild-type virus following high-dose intravenous infection. However our data (Lewicki *et al.*, manuscript submitted) indicate that a GP1 and GP2 double escape variant of LCMV ARM is cleared with similar kinetics to wild-type virus following high-dose infection. If it is assumed that escape mutations must be present in all three known H-2^b CTL epitopes of LCMV to produce a virus with significantly different *in vivo* survival properties from those of the wild-type virus, the probability that a biologically meaningful CTL escape mutant virus will be generated is extremely low, in the range of 2.147×10^{-6}%. Indeed, when mutation of the whole virus genome is considered, the probability is several magnitudes less.

Can biologically meaningful CTL escape variants be generated *in vivo?* Pircher and associates (Pircher *et al.*, 1990) showed that, during acute infection, LCMV escaped immunosurveillance *in vivo* by virus-specific CTL after mutations altered the GP1 epitope in mice made transgenic for and expressing a TCR recognizing the GP1 epitope. This TCR is homogeneous and uses Vα2 and Vβ8. This observation would parallel our finding that selection of mutants by CTL exerting selective pressure on a single peptide epitope may not be uncommon, i.e., likely occurs at a frequency of 2%. However, in C57BL/6

FIG. 5. Stereo diagrams of models for LCMV-derived peptides GP1 (33–41, KAVYNFATC) and NP (396–404, FQPQNGQFI) bound to H-$2D^b$. The solvent-exposed surface of H-$2D^b$ is rendered in blue and the GP1 and NP peptides in white and yellow, respectively. The orientations shown place the amino termini of the peptides at the top of the pictures. The mutated amino acids discussed in the text are noted by numbers in parentheses and are based on the original peptide numbering schemes. For both of the modeled peptides the first three residues and the conserved anchor residue in the fifth position are highly buried. The two residues that are mutated are solvent exposed and would, based on these models, be able to interact directly with TCR molecules.

TABLE 3

Abrogation of CTL Recognition of LCMV GP1 and NP Peptides Restricted by D^b Molecules Following Ala Substitution of Authentic Peptide Residue

	Residue 1 2 3 4 5 6 7 8 9 GP1 K A V Y N F A T C				1 2 3 4 5 6 7 8 9 NP F Q P Q N G Q F I		
Ala substitution in residue No.		Peptide 10^{-} *M*	% Specific CTL lysis	Ala substitution in residue No.		Peptide 10^{-} *M*	% Specific CTL lysis
3	V → A	6.0	97	3	P → A	6.0	12
3		7.0	76	3		7.0	10
3		8.0	25	3		8.0	10
5	N → A	6.0	0	5	N → A	6.0	3
5		7.0	0	5		7.0	2
5		8.0	0	5		8.0	0
6	F → A	6.0	8	6	G → A	6.0	2
6		7.0	0	6		7.0	2
6		8.0	0	6		8.0	2
8	T → A	6.0	0	8	F → A	6.0	10
8		7.0	0	8		7.0	7
8		8.0	3	8		8.0	10
9	C → A	6.0	100	9	I → A	6.0	82
9		7.0	100	9		7.0	100
9		8.0	100	9		8.0	100

Note. Computer graphic analysis suggested that residues 1, 2, 3, 5, and 9 are anchored in the MHC groove while residues 4, 6, 7, and 8 face outward toward the TCR. Authentic peptides or peptides with Ala substitutions were incubated with uninfected ^{51}Cr-labeled H-2^b MC57 cells and then reacted at 37° with CTLs that specifically recognize either the authentic GP1 or the authentic NP peptide. After 5–6 hr, the ^{51}Cr counts released in the supernatant were quantitated. Similar results were obtained in other experiments. Percentage of specific CTL lysis indicates the % specific ^{51}Cr release in the presence of variant peptides expressed as a percentage of the % specific ^{51}Cr release in the presence of the authentic peptide. Numbers represent the mean of values from triplicate samples. Variance was <10%.

mice not just a single CTL epitope but at least three occur. Further, our (Oldstone *et al.,* 1986; Tishon *et al.,* 1993) and other (Ahmed *et al.,* 1987) laboratories have shown that MHC-restricted LCMV-specific CTL adoptively transferred to LCMV persistently infected mice effectively clear infectious virus and viral nucleic acid sequences from their sera and tissues. However, dependent on the MHC haplotype, about 2 to 15% of such mice do, over time, revert to a state of persistent infection. Study of such revertant viruses isolated by Ahmed (1994) indicated that the viruses recovered displayed no mutation in viral peptides comprising CTL epitopes; instead the defect was elsewhere in handling of the virus by macrophages. Our unpublished data are similar. Further, we recently observed (Oldstone *et al.,* 1994) that adoptive transfer of LCMV CTL into persistently infected H-2^b mice that were also genetically deficient in CD4 cells, routinely resulted in clearance of infectious virus from all recipients within 14 to 21 days, but by 28–35 days all mice again had persistent infections. Analysis of viruses isolated from these mice also failed to reveal any dysfunction in generation of CTL responses to any of the three D^b-restricted CTL epitopes making it unlikely that CTL escape variants were generated. Viruses isolated from persistently infected mice differed from the wt virus in that the former were less likely to be inactivated by IFN-γ-activated macrophages than the wt LCMV ARM. Thus, there is no evidence, at present, to indicate that biologically meaningful CTL escape variants can be selected and generated *in vivo* during acute or persistent infection in a LCMV-infected host having a normal T cell repertoire and TCR diversity, although theoretically this could occur as it did under nonphysiologic conditions (Pircher *et al.,* 1990). Earlier, analysis of LCMV CTL clones generated to the three D^b or to the immunodominant single L^d CTL peptide epitopes showed a diversity in TCR responses (Yanagi *et al.,* 1990; Joly *et al.,* 1989; Horwitz *et al.,* 1994), indicating another reason for the low probability that CTL escape variants develop in living animals.

Persistent viral infections are not uncommon in humans. For such persistence to occur, viruses must accomplish two tasks. The first is to devise a strategy(ies) of replication that ensures a nonlytic phenotype, a process that likely requires the virus' active participation as well as inactivation of the infected cell's apoptosis or suicidal tendencies (Oldstone, 1991; Razvi and Welsh, 1994; Chou and Roizman, 1992). Second, the virus must actively interfere with the host's antiviral immune response, whose purpose is to generate effector cells and/or molecules that recognize virus and virally infected cells as foreign (Whitton and Oldstone, 1995; Oldstone, 1991; Koup, 1994). One way to escape from immune sur-

veillance may occur through the selection of CTL escape viruses. Although evidence is presented here and elsewhere (Aebischer *et al.,* 1991) for *in vitro* selection of CTL escape variants as a strategy of circumventing the immune response, and this device has also been postulated for *in vivo* selection in several RNA and DNA virus infections of man (de Campos-Lima *et al.,* 1993, 1994; Bertoletti *et al.,* 1994; Klenerman *et al.,* 1994; Phillips *et al.,* 1991), it would appear that the likelihood of this mechanism occurring is of low probability because of the multiple MHC haplotypes in any individual and the several virus peptide epitopes restricted by MHC (H-2 K, D or MHC A, B, C) alleles.

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REFERENCES

Aebischer, T., Moskophidis, D., Rohrer, U. H., Zinkernagel, R. H., and Hengartner, R. (1991). In vitro selection of lymphocytic choriomeningitis virus escape mutants by cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA* **88,** 11047–11051.

Ahmed, R. (1994). Evasions of host immune response-II. FASEB Conf., June 19–24.

Ahmed, R., Jamieson, B. D., and Porter, D. (1987). Immune therapy of a persistent and disseminated viral infection. *J. Virol.* **61,** 3920–3929.

Bertoletti, A., Costanzo, A., Chisari, F. V., Levrero, M., Artini, M., Sette, A., Penna, A., Giuberti, T., Fiaccadori, F., and Ferrari, C. (1994). Cytotoxic T lymphocyte response to a wild type hepatitis B virus epitope in patients chronically infected by variant viruses carrying substitutions within the epitope. *J. Exp. Med.* **180,** 933–943.

Bertoletti, A., Sette, A., Chisari, F. V., Penna, A., Levrero, M., De Carli, M., Fiaccadori, F., and Ferrari, C. (1994). Natural variants of cytotoxic epitopes are T cell receptor antagonists for anti-viral cytotoxic T cells. *Nature (London)* **369,** 407–410.

Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L., and Wiley, D. C. (1987). Structure of the human class I histocompatibility antigen, HLA-A2. *Nature (London)* **329,** 506–511.

Braciale, T. J. (1992). Antigen processing for presentation by MHC class I molecules. *Curr. Opinion Immunol.* **4,** 59–62.

Braciale, T. J., and Braciale, V. L. (1992). Viral antigen presentation and MHC assembly. *Semin. Immunol.* **4,** 81–84.

Buchmeier, M. J., Lewicki, H. A., Tomori, O., and Oldstone, M. B. A. (1981). Monoclonal antibodies to lymphocytic choriomeningitis virus and Pichinde viruses: Generation, characterization, and cross-reactivity with other arenaviruses. *Virology* **113,** 73–85.

Byrne, J. A., and Oldstone, M. B. A. (1984). Biology of cloned cytotoxic T lymphocytes specific for lymphocytic choriomeningitis virus: Clearance of virus in vivo. *J. Virol.* **51,** 682–686.

Chou, J., and Roizman, B. (1992). The γ1 34.5 gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programmed cell death in neuronal cells. *Proc. Natl. Acad. Sci. USA* **89,** 3266–3270.

de Campos-Lima, P.-O., Gavioli, R., Zhang, Q.-J., Wallace, L. E., Dolcetti, R., Rowe, M., Rickinson, A. B., and Masucci, M. G. (1993). HLA-A11 epitope loss isolates of Epstein–Barr virus from a highly A11+ population. *Science* **260,** 98–100.

de Campos-Lima, P.-O., Levitsky, V., Brooks, J., Lee, S. P., Hu, L. F., Rickinson, A. B., and Masucci, M. G. (1994). T cell responses and virus evolution: Loss of HLA A11-restricted CTL epitopes in Epstein–Barr virus isolates from highly A11-positive populations by selective mutation of anchor residues. *J. Exp. Med.* **179,** 1297–1305.

Falk, K., Rotzschke, O., Stevanovic, S., Jung, G., and Rammensee, H. G. (1991). Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature (London)* **351,** 290–296.

Fremont, D. H., Matsumura, M., Stura, E. A., Peterson, P. A., and Wilson, I. A. (1992). Crystal structures of two viral peptides in complex with murine MHC class I H-2K^b. *Science* **257,** 919–927.

Gairin, J. E., and Oldstone, M. B. A. (1992). Design of high-affinity major histocompatibility complex-specific antagonist peptides that inhibit cytotoxic T-lymphocyte activity: Implications for control of viral disease. *J. Virol.* **66,** 6755–6762.

Gairin, J. E., and Oldstone, M. B. A. (1993). Virus and cytotoxic T lymphocytes: Crucial role of viral peptide secondary structure in major histocompatibility complex class I interactions. *J. Virol.* **67,** 2903–2907.

Gairin, J. E., Mazarguil, H., Hudrisier, D., and Oldstone, M. B. A. (1995). Optimal lymphocytic choriomeningitis virus sequences restricted by H-2D^b-MHC class I molecules and presented to cytotoxic T lymphocytes. *J. Virol.* **69,** 2297–2305.

Garboczi, D. N., Madden, D. R., and Wiley, D. C. (1994). Five viral peptide-HLA-A2 co-crystals, simultaneous space group determination and x-ray data collection. *J. Mol. Biol.* **239,** 581–587.

Horwitz, M. S., Yanagi, Y., and Oldstone, M. B. A. (1994). T-cell receptors from virus specific cytotoxic T lymphocytes recognizing a single immunodominant nine amino acid viral epitope show marked diversity. *J. Virol.* **68,** 352–357.

Joly, E., Mucke, L., and Oldstone, M. B. A. (1991). Viral persistence in neurons explained by lack of major histocompatibility complex class I expression. *Science* **253,** 1283–1285.

Joly, E., Salvato, M., Whitton, J. L., and Oldstone, M. B. A. (1989). Polymorphism of cytotoxic T-lymphocyte clones that recognize a defined nine amino acid immunodominant domain of lymphocytic choriomeningitis virus glycoprotein. *J. Virol.* **63,** 1845–1851.

Jones, T. A., Cowan, S., Zou, J. Y., and Kjeldgaard, M. (1991). Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr., Sect. A* **47,** 110–119.

Klavinskis, L. S., Whitton, J. L., Joly, E., and Oldstone, M. B. A. (1990). Vaccination and protection from a lethal virus infection: Identification, incorporation, and use of a cytotoxic T lymphocyte glycoprotein epitope. *Virology* **178,** 393–400.

Klenerman, P., Rowland-Jones, S., McAdam, S., Edwards, J., Daenke, S., Lalloo, D., Köppe, B., Rosenberg, W., Boyd, D., Edwards, A., Giangrande, P., Phillips, R. E., and McMichael, A. J. (1994). Cytotoxic T-cell activity antagonized by naturally occurring HIV-1 gag variants. *Nature (London)* **369,** 403–407.

Koup, R. A. (1994). Virus escape from CTL recognition. *J. Exp. Med.* **180,** 779–782.

Lampson, L. A. (1987). Molecular basis of the immune response to neural antigens. *Trends Neurosci.* **10,** 211–216.

Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thorton, J. M. (1993). PROCHECK: A program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.* **26,** 283–291.

Laver, W. G., and Webster, R. G. (1973). Studies on the origin of pandemic influenza. III. Evidence implicating duck and equine influenza viruses as possible progenitors of the Hong Kong strain of human influenza. *Virology* **51,** 383–390.

Laver, W. G., Air, G. M., Webster, R. G., Gerhard, W., Ward, C. W., and Dopheide, T. A. A. (1979). Antigenic drift in type A influenza virus: Sequence differences in the hemagglutinin of Hong Kong (H3N2) variants selected with monoclonal hybridoma antibodies. *Virology* **98,** 226–237.

Lill, N. L., Tevethia, M. J., Hendrickson, W. G., and Tevethia, S. S. (1992). Cytotoxic T lymphocytes (CTL) against a transforming gene product select for transformed cells with point mutations within sequences encoding CTL recognition epitopes. *J. Exp. Med.* **176,** 449–457.

Lin, Y., and Askonas, B. A. (1981). Biological properties of an influenza A virus specific killer T cell clone. Inhibition of virus replication in vivo and induction of delayed hypersensitivity reactions. *J. Exp. Med.* **154,** 225–234.

Ljunggren, H. G., Stam, N. J., Olhen, C., Neefjes, J. J., Hoglund, P., Heemels, P., Bastin, J., Schumacher, T. N., Townsend, A., and Karre, K. (1990). Empty MHC class I molecules come out of the cold. *Nature (London)* **346,** 476–480.

Madden, D. R., Garboczi, D. N., and Wiley, D. C. (1993). The antigenic identity of peptide–MHC complexes: A comparison of the conformations of five viral peptides presented by HLA-A2. *Cell* **75,** 693–708.

Matsumura, M., Fremont, D. H., Peterson, P. A., and Wilson, I. A. (1992). Emerging principles for the recognition of peptide antigens by MHC class I molecules. *Science* **257,** 927–934.

Merrifield, R. B. (1963). Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* **85,** 2149.

Moskophidis, D., and Zinkernagel, R. M. (1995). Immunobiology of cytotoxic T-cell escape mutants of lymphocytic choriomeningitis virus. *J. Virol.* **69**(4), 2187–2193.

Narayan, O., Griffin, D. E., and Chase, J. (1977). Antigenic shift of visna virus in persistently infected sheep. *Science* **197,** 376–378.

Oldstone, M. B. A. (1991). Molecular anatomy of viral persistence. *J. Virol.* **65,** 6381–6386.

Oldstone, M. B. A. (1994). Cytotoxic T-lymphocytes in human viral and malaria infections. *In* "Current Topics in Microbiology and Immunology" (M. B. A. Oldstone, Ed.), Vol. 189. Springer-Verlag, Berlin/Heidelberg, Germany.

Oldstone, M. B. A., Blount, P., Southern, P. J., and Lampert, P. J. (1986). Cytoimmunotherapy for persistent virus infection: Unique clearance pattern from the central nervous system. *Nature (London)* **321,** 239–243.

Oldstone, M. B. A., Nerenberg, M., Southern, P., Price, J., and Lewicki, H. (1991). Virus infection triggers insulin dependent diabetes mellitus in a transgenic model: Role of anti-self (virus) immune response. *Cell* **65,** 319–331.

Oldstone, M. B. A., Tishon, A., and Lewicki, H. (1994). Generation of unique viral variants from persistently infected CD4 genetically deficient escape inactivation by macrophages. Submitted for publication.

Oldstone, M. B. A., Tishon, A., Eddleston, M., de la Torre, J. C., McKee, T., and Whitton, J. L. (1993). Vaccination to prevent persistent viral infection. *J. Virol.* **67,** 4372–4378.

Oldstone, M. B. A., Tishon, A., Geckeler, R., Lewicki, H., and Whitton, J. L. (1992). A common antiviral cytotoxic T-lymphocyte epitope for diverse major histocompatibility haplotypes: Implications for vaccination. *Proc. Natl. Acad. Sci. USA* **89,** 2752–2755.

Oldstone, M. B. A., Whitton, J. L., Lewicki, H., and Tishon, A. (1988). Fine dissection of a nine amino acid glycoprotein epitope, a major determinant recognized by lymphocytic choriomeningitis virus specific class I restricted H-2D^b cytotoxic T lymphocytes. *J. Exp. Med.* **168,** 559–570.

Phillips, R. E., Rowland-Jones, S., Nixon, D. F., Gotch, F. M., Edwards, J. P., Ogunlesi, A. O., Elvin, J. G., Rothbard, J. A., Bangham, Ch. R. M., Rizza, Ch. R., and McMichael, A. J. (1991). Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition. *Nature (London)* **354,** 453–459.

Pircher, H., Moskophidis, D., Rohrer, U., Bürki, K., Hengartner, H., and Zinkernagel, R. M. (1990). Viral escape by selection of cytotoxic T cell-resistant virus variants in vivo. *Nature (London)* **346,** 629–633.

Razvi, E. S., and Welsh, R. M. (1995). Apoptosis in viral infections. *Adv. Virus. Res.,* in press.

Salvato, M., Borrow, P., Shimomaye, E., and Oldstone, M. B. A. (1991). Molecular basis of viral persistence: A single amino acid change in the glycoprotein of lymphocytic choriomeningitis virus is associated with suppression of the antiviral cytotoxic T lymphocyte response and establishment of persistence. *J. Virol.* **65,** 1863–1869.

Tishon, A., Eddleston, M., de la Torre, J. C., and Oldstone, M. B. A. (1993). Cytotoxic T lymphocytes cleanse viral gene products from individually infected neurons and lymphocytes in mice persistently infected with lymphocytic choriomeningitis virus. *Virology* **197,** 463–467.

Townsend, A. R. M., Rothbard, J., Gotch, F. M., Bahadur, G., Wraith, D., and McMichael, A. J. (1986). The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell* **44,** 959–968.

van Bleek, G. M., and Nathanson, S. G. (1990). Isolation of an endogenously processed immunodominant viral peptide from the class I H-2K^b molecule. *Nature* **348,** 23–26.

Whitton, J. L., and Oldstone, M. B. A. (1989). Class I MHC can present an endogenous peptide to cytotoxic T lymphocytes. *J. Exp. Med.* **170,** 1033–1038.

Whitton, J. L., and Oldstone, M. B. A. (1995). General virology: The immune response to viruses. *In* "Virology" (B. N. Fields, D. M. Knipe, P. M. Howley, *et al.,* Eds.), 3rd ed. Raven Press, New York, in press.

Whitton, J. L., Sheng, N., Oldstone, M. B. A., and McKee, T. A. (1993). A "string-of-beads" vaccine, comprising linked minigenes, confers protection from lethal-dose virus challenge. *J. Virol.* **67,** 348–352.

Whitton, J. L., Southern, P. J., and Oldstone, M. B. A. (1988). Analyses of the cytotoxic T lymphocyte responses to glycoprotein and nucleoprotein components of lymphocytic choriomeningitis virus. *Virology* **162,** 321–327.

Whitton, J. L., Tishon, A., Lewicki, H., Gebhard, Cook, T., Salvato, M., Joly, E., and Oldstone, M. B. A. (1989). Molecular analyses of a five amino acid cytotoxic T lymphocyte (CTL) epitope: An immunodominant region which induces nonreciprocal CTL cross-reactivity. *J. Virol.* **63,** 4303–4310.

Wiktor, T. J., and Koprowski, H. (1978). Monoclonal antibodies against rabies virus produced by somatic cell hybridization: Detection of antigenic variants. *Proc. Natl. Acad. Sci. USA* **75,** 3938–3942.

Yanagi, Y., Maekawa, R., Cook, T., Kanagawa, O., and Oldstone, M. B. A. (1990). Restricted V-segment usage in T-cell receptors from cytotoxic T lymphocytes specific for a major epitope of lymphocytic choriomeningitis virus. *J. Virol.* **64,** 5919–5926.

Yanagi, Y., Tishon, A., Lewicki, H., Cubitt, B. A., and Oldstone, M. B. A. (1992). Diversity of T-cell receptors in virus-specific cytotoxic T lymphocytes recognizing three distinct viral epitopes restricted by a single major histocompatibility complex molecule. *J. Virol.* **66,** 2527–2531.

Yewdell, J. W., Webster, R. G., and Gerhard, W. U. (1979). Antigenic variation in three distinct determinants of an influenza type A haemagglutinin molecule. *Nature (London)* **279,** 246–248.

Young, A. C., Zhang, W., Sacchettini, J. C., and Nathenson, S. G. (1994). The three-dimensional structure of H-2D^b at 2.4 Å resolution: Implications for antigen-determinant selection. *Cell* **76,** 39–50.

Zinkernagel, R. M., and Doherty, P. C. (1974). Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature (London)* **248,** 701–702.

Zinkernagel, R. M., and Welsh, R. M. (1976). H-2 compatibility requirement for virus specific T cell-mediated effector functions in vivo. I. Specificity of T cells conferring antiviral protection against LCMV is associated with H-2K and H-2D. *J. Immunol.* **117,** 1495–1502.