

USE OF SYNTHETIC PEPTIDES OF INFLUENZA  
NUCLEOPROTEIN TO DEFINE EPITOPES RECOGNIZED BY  
CLASS I-RESTRICTED CYTOTOXIC T LYMPHOCYTES

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CTL are partly responsible for the immune response to influenza viruses in both mouse and man. The majority of CTL detectable *in vivo* and early in polyclonal cultures *in vitro*, crossreact on target cells infected with all strains of A viruses but not B viruses (1, 2). This crossreactive CTL response may play a role in the heterotypic protection observed in rodents and man after infection by serologically distinct influenza A strains (3, 4).

The identification of the conserved viral molecules that are recognized by crossreactive CTL has depended on rDNA techniques to express individual viral proteins in cell lines *in vitro*. In both mice and man the predominant conserved epitopes turn out to be on the nonglycosylated proteins of the virus (5–9). The nucleoprotein (NP)<sup>1</sup> is a major antigen in H-2k, -d, and -b strains of mice (5, 6) and in the majority of humans (7). PB1, PB2, PA, NS1, and M1 also have been shown to be recognized by class I-restricted CTL (7, 8). The CTL response to NP appears to be under strict genetic control in both mice and man, responder status being conferred by single polymorphic class I genes in each case (10–12).

Recent efforts (10) to map the epitopes recognized to regions of the NP sequence using deletion mutants of the NP gene have shown that the immunodominant epitopes lie in different regions of the molecule in H-2b and -k strains of mice. These experiments also showed that the recognition of NP by CTL was not dependent on a definable signal sequence in the molecule, and led to the suggestion that NP may be recognized after degradation in the target cell. Further work (13) demonstrated that an epitope mapped to a short region of NP that had undergone genetic change since 1934 could be replaced *in vitro* with a short peptide (amino acids 366–379). Two mutually exclusive epitopes were thus defined using CTL clones that differentiated between two groups of A viruses isolated between 1934–1943 and 1946–1968. During the course of these experiments an additional epitope recognized by HLA-B37-restricted human CTL was defined with a second NP peptide (335–349) (13).

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<sup>1</sup>Abbreviations used in this paper: HAU, hemagglutinin units; K/T, killer-to-target ratio; NP, influenza virus nucleoprotein.

In this paper we extend these findings by showing that the two conserved NP epitopes recognized by CBA (H-2k) and C57BL/10 (H-2b) mice defined in the deletion analysis (10) can also be replaced with peptides *in vitro*. We examine the details of the specificity of two CTL clones from C57BL/10 mice that recognize different epitopes on the peptide 365–379 through the same restriction element (Db), and show that recognition by clone F5 (5, 10, 13) is sensitive to the single conserved substitution Asp to Glu at position 372 in NP. Finally, we demonstrate that the complete NP protein is inefficient at sensitizing target cells for lysis by CTL in comparison to peptide. These results strengthen the hypothesis that a ubiquitous mechanism exists by which newly synthesized viral (and possibly host) proteins are degraded and presented to CTL in association with class I molecules of the MHC.

### Materials and Methods

*Mice.* 3–6-mo-old C57BL/10 female mice used as responding cell donors were obtained from National Institute of Medical Research, London, United Kingdom.

*In Vivo Priming of Mice.* Mice were anesthetized with ether and primed by intranasal infection with 5 hemagglutinin units (HAU) of E61-13-H17 virus or by intravenous inoculation with 100 HAU of A/PR/8/34 virus as infectious allantoic fluid diluted in 50  $\mu$ l PBS as described (14).

*Influenza Virus Strains.* The recombinant A viruses X31 (15) and E61-13-H17 (reference 16 where this virus is referred to as recombinant 33) differ only in the origin of their genes for nucleoprotein. E61-13-H17 contains the 1968 NP gene from A/HK/8/68, and X31 contains the 1934 NP gene from A/PR/8/34. Virus was grown in the allantoic sacs of 11-d-old embryonated chicken eggs and stored as infectious allantoic fluid at  $-70^{\circ}\text{C}$ .

*Peptide Synthesis.* Peptides were synthesized by solid-phase techniques on a peptide synthesizer (No. 430A Applied Biosystems, Inc., Foster City, CA) as described (13). The purity of the crude product was determined by HPLC on a C-18 reverse-phase column. All the peptides described here contained  $>90\%$  of the desired product.

*Transfected L Cells.* L cells were cotransfected with the Db class I gene and the BAM577/793 deletion mutant of the NP cDNA (under the transcriptional control of the Herpes Simplex virus thymidine kinase promoter) exactly as described previously (10). The BAM577/793 mutant expresses amino acids 1–87, 160–498 of NP and its construction has been described previously (17). The L cell transfectants expressing the full length NP (amino acids 1–498), and the deletion mutants PVU705 (amino acids 1–130), PVU1295 (amino acids 1–327), and IMP1295 (amino acids 1, 2, 328–498) have been described (10).

*Peptide-pulsed Target Cells.* L cells transfected with the gene for Db (L/Db) were labeled with  $^{51}\text{Cr}$  as described (14), then resuspended in RPMI 1640 medium containing 10% FCS and peptide at 30  $\mu\text{M}$ /liter for 1 h. They were then washed four times as described (13) before use in the cytotoxicity assay.

*Purification of Influenza Nucleoprotein Produced in Escherichia coli.* The NP was expressed in *E. coli* as a fusion protein with the  $\lambda$  Cro gene product (18). The final NP product contains an  $\text{NH}_2$ -terminal extension of 32 heterologous amino acids, which do not interfere with its biological activities (19). It was purified by immune-affinity chromatography as described (19), or by ion exchange chromatography as follows.

Briefly, induced bacterial cultures were pelleted, resuspended in 10 mM Tris, HCl, pH 7.0, 1 mM PMSF (Sigma Chemical Co., St. Louis, MO) and were lysed by sonication. The extract was clarified by centrifugation and applied to a column of DEAE-Sepharose (Bio-Rad Laboratories, Richmond, CA). NP was eluted from the column using a linear gradient of 0–0.5 M NaCl, and fractions containing NP (detected by gel electrophoresis) were pooled, and made up to 50 mM Tris-acetate, pH 5.5. A precipitate that formed at  $0^{\circ}\text{C}$

was removed by centrifugation and the supernatant was dialyzed against 10 mM Tris, pH 7.0/1 mM PMSF. The extract was applied to a column of P11-phosphocellulose (Whatman Inc., Clifton, NJ) and NP was eluted with a linear gradient of 0–1 M NaCl. NP prepared in this way was judged to be >90% pure by gel electrophoresis and was stored for use in 0.5 M NaCl at  $-20^{\circ}\text{C}$ . The concentration of the purified product ( $M_r$  60,000) was measured by Bradford's method (20). For some experiments, the purified recombinant NP was denatured with 7 M guanidine-HCl followed by reduction and alkylation as described by Campbell et al. (21) before testing in the cytotoxicity assay.

*Polyclonal Cytotoxic T Cell Cultures.* The methods used to initiate and maintain cultures of influenza specific CTL have been described (14).

*Polyclonal CBA (H-2k) CTL.* Male CBA mice were primed by intravenous injection of 100 HAU of A/PR/8/34 virus. 2 wk later they were killed and cultures of their spleen cells were initiated in vitro by stimulation with A/PR/8/34 virus as described (14), except that the supernatant from PMA-stimulated EL4 cells (22) (EL4 supernatant) was used as a source of T cell growth factors after 1 wk in vitro. After three weekly stimulations with A/PR/8/34 virus-infected feeder cells the culture was tested in the cytotoxicity assay.

*CTL Clone 11.* Clone 11 was derived from a C57BL/10 mouse primed intranasally with E61-13-H17 (1968 NP). Established polyclonal CTL lines were raised, as described previously (14), and maintained on X31 (which expresses the 1934 NP)-infected feeder cells. The CTL were cloned by limiting dilution, as described (23), using as a source of growth factors the supernatant from PMA-stimulated EL4 cells (22) diluted to 5–10% vol/vol. Once the specificities of the clones were known, the cultures were maintained on peptide-pulsed, irradiated (2,000 rad) spleen cells. Irradiated spleen cells were pulsed with 100  $\mu\text{g}/\text{ml}$  of appropriate peptide diluted in RPMI/10% FCS for 1 h at  $37^{\circ}\text{C}$ , then they were washed three times in PBS and used as feeder cells as described previously (13).

*CTL Clone F5.* F5 is a Db-restricted C57BL/6 CTL clone specific for 1968 NP and has been described previously (5, 10, 13).

*$^{51}\text{Chromium}$ -release Assay.* A standard procedure was used (24) for target cells either infected with influenza virus, transfected with NP gene fragments, or pulsed with peptide and washed before use in the assay (13), (Figs. 1–3).  $^{51}\text{Cr}$  release was calculated as follows: % specific release = [(release by CTL – medium release)/(2.5% Triton release – medium release)]  $\times$  100.

Each experimental point was measured in duplicate against quadruplicate controls in medium alone. Spontaneous  $^{51}\text{Cr}$  release by target cells in medium in the absence of CTL was <18% of that released by Triton X-100.

*Dose Response Titrations of Peptides.* For all of the dose-response experiments (Figs. 3–6), L cells transfected with the class I gene Db were used (5) as target cells. Peptides in RPMI 10 or NP in isotonic saline solution were dispensed in round-bottomed 96-well Sterilin microtiter plates, as described (13), in 0.05 ml, and a series of dilutions were made over a range optimized in previous experiments.  $^{51}\text{Cr}$ -labeled LD<sup>b</sup> cells ( $10^4$ ) in 0.05 ml were added to each well followed by a fixed number of CTL in 0.1 ml (to give the CTL/target cell ratios described in the legends to the figures). The 6-h assay was then performed as previously described (13, 14). The results in Figs. 3–6 were calculated in the following way to normalize the titrations to the 0% line: [(Release in the presence of CTL and peptide – release by CTL alone)/(2.5% Triton release – release by CTL alone)]  $\times$  100.

The release of  $^{51}\text{Cr}$  from target cells in the presence of CTL (at the K/T ratios shown in Figs. 3–6) but no peptide was between 16–36% of the counts released by Triton. These values were slightly greater than the spontaneous release of  $^{51}\text{Cr}$  from target cells in the absence of peptide or CTL (14–29%). Within the dose ranges shown none of the peptides alone had any detectable effect on the spontaneous release of  $^{51}\text{Cr}$  from target cells.

## Results

*The Major Conserved Epitope of NP Recognized by CBA (H-2k) CTL Is Defined by Peptide 50–63.* We have previously shown (10) that a major proportion of CBA

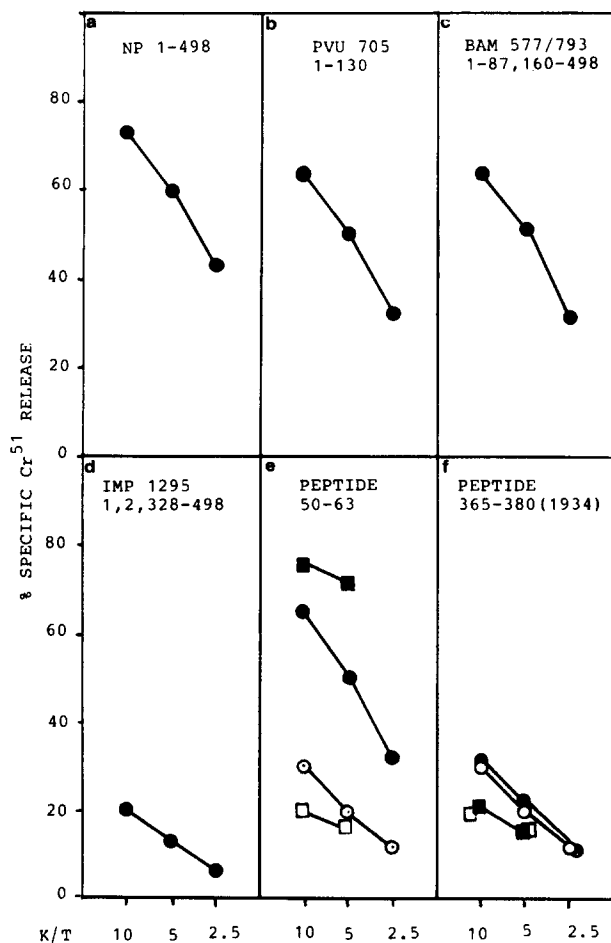


FIGURE 1. Recognition by polyclonal CBA CTL line of L/D<sup>b</sup> target cells transfected with full length or truncated NP genes (*a-d*) and L/D<sup>b</sup> cells pulsed with peptides (*e* and *f*). The CTL line was derived from a CBA mouse as described in Materials and Methods. After 2 wk of stimulation in vitro with A/PR/8/34 virus, the CTL line was split, and one-half was stimulated with feeder cells pulsed with 30  $\mu$ M/liter of peptide 50-63, and the other half with A/PR/8/34 virus. (●) CTL stimulated three times with A/PR/8/34 virus and tested on transfected or peptide-treated L/D<sup>b</sup> target cells; (○) the same effector cells on untreated L/D<sup>b</sup> target cells; (■) CTL stimulated twice with A/PR/8/34 virus, and once with spleen cells pulsed with peptide 50-63, (□) the same CTL tested on untreated L/D<sup>b</sup> target cells.

and C3H (H-2k) CTL generated in secondary in vitro responses to influenza are specific for a conserved epitope of NP localized to the NH<sub>2</sub>-terminal region of the molecule comprising amino acids 1-130. Fig. 1 shows an extension of this analysis using a further transfected target cell expressing a deletion mutant of NP (BAM 577/793; see reference 17) which localized the predominant epitope to the NH<sub>2</sub>-terminal 87 amino acids of NP.

Recently, the observation has been made (25) that epitopes recognized by T cells that can be defined with synthetic peptides in vitro tend to contain amino acids with defined physical properties in the following order more often than would be expected by chance: (Charged or Glycine) - (Hydrophobic) - (Hydrophobic) - (Charged or Polar).

The NH<sub>2</sub>-terminal 87 amino acids of NP contain three such regions ("motifs") of sequence. The first peptide synthesized to contain one of these was composed of amino acids 50-63 (Table I), which contains the commonly observed motif in the sequence Arg-Leu-Ile-Gln (see reference 26 for the NP sequence from A/NT/60/68). The currently updated predictive program described by De Lisi

TABLE I  
*Summary of the NP Peptides Recognized by Class I-restricted CTL*

Sequence	Specificity	Restriction element	Reference
365-380 (IASNENMETMESSTLE)	1934-1943	H-2D <sup>b</sup>	13
365-379 (IASNENMDAMESSTL)	1946-1968	H-2D <sup>b</sup>	13
365-379 (IASNENMDAMESSTL)	All A strains	H-2D <sup>b</sup>	This paper
50-63 (SDYEGRLIQNSLTI)	All A strains	H-2K <sup>k</sup>	This paper
335-349 (SAAFEDLRVLSFIRG)	All A strains	HLA-B37	13

For the sequences of the nucleoproteins from 1968 and 1934 see references 26 and 28.

and Berzofsky (27) selects 65 of the 87 NH<sub>2</sub>-terminal residues, which include the sequence 50-63.

CTL recognition of this peptide was readily detected in polyclonal cultures with NP-specific activity from CBA (H-2k) mice (Fig. 1). CBA cultures did not recognize the peptide 365-379, which was recognized efficiently by C57BL (H-2b) CTL (described below). CTL with specificity for peptide 50-63 could be rapidly enriched in polyclonal cultures by restimulation with peptide-pulsed feeder cells and a source of T cell growth factors as described (22) (Fig. 1e). The MHC restriction for this peptide can be inferred, from the studies of Pala and Askonas, to be through Kk (11); they have shown that all NP-specific CTL in CBA mice are restricted through the class I molecule Kk.

*The Major Conserved Epitope on NP Recognized by C57BL (H-2b) Mice Defined with Peptide 365-379.* Within the polyclonal CTL response to influenza in C57BL there exists a predominant population that recognize target cells expressing NP in isolation after DNA transfection (5). The majority of these NP specific CTL are crossreactive for all A strains of influenza that have been associated with epidemics in man (5, 10). There also exists a minor population of H-2b CTL that recognize a genetically variable region of NP that can be defined with subgroups of influenza A isolates (5, 14). Recently (13), CTL clones specific for the latter determinant were shown to recognize a synthetic peptide representing the amino acids 365-379 of NP. Two noncrossreacting epitopes were demonstrated, one expressed in viruses isolated between 1934 and 1943, the other differing by two amino acids and expressed in viruses isolated between 1946 and 1968 (13, 14). These results are summarized in Table I.

The majority of the NP-specific CTL detected in polyclonal cultures from H-2b mice, which are crossreactive between viral strains, can be shown to recognize the same region of NP sequence as the subgroup-specific CTL by using target cells transfected with appropriately truncated NP genes (10). We have now screened such cultures on target cells treated with peptides derived from the NP sequence.

Table II shows the results from a polyclonal culture initiated and maintained as described (14). Most of the NP-specific CTL activity is directed at the COOH-terminal region of NP defined by transfectant IMP1295 (which expresses amino acids 1, 2, 328-498). When tested on peptide-treated target cells, a detectable proportion of the NP-specific activity was found to be directed at peptide 365-379 derived from either the 1934 (28) or 1968 (26) NP sequence. To ensure

TABLE II  
*Polyclonal C57BL/10 CTL Activity Showing Crossreactive Specificity  
 for Peptide 365-379*

Target cell	Specific lysis*
	%
Infected	
AX31 (1934 NP)-infected LD <sup>b</sup> cells	69.0
LD <sup>b</sup> uninfected	0.5
Peptide pulsed	
1934; 365-380 peptide-pulsed LD <sup>b</sup>	40.0
1968; 365-379 peptide-pulsed LD <sup>b</sup>	56.5
1968; 335-349 peptide-pulsed LD <sup>b</sup>	9.5
Transfected	
PVU 1295 (1-327)	2.5
IMP 1295 (1, 2, and 328-498)	52.5

\* Obtained in standard <sup>51</sup>Cr-release assay using a killer-to-target ratio (K/T) of 2.5:1 as described in Materials and Methods. C57BL/10 mice were primed with E61-13-H17 virus (1968 NP) and maintained in vitro by repeated stimulation with X31 virus (1934 NP) as described (14).

that this crossreactive specificity existed at the clonal level the polyclonal culture was cloned by limiting dilution and the resulting CTL clones were tested on peptide-pulsed and transfected target cells. Each of the four clones isolated that recognized NP from both 1934 and 1968 viruses were found to recognize peptide 365-379 derived from either sequence.

The results for clone 11 are shown in Fig. 2. *a-d* show the specificity of clone 11 on target cells transfected with fragments of the 1968 NP gene. *e* and *f* show the recognition of peptide-pulsed target cells. Target cells not transfected with the gene coding for the class I molecule Db were not recognized (data not shown).

*Comparison of the Fine Specificities of Clones 11 and F5.* In previous work we defined clone F5 as specific for the 1968 NP sequence 365-380 (Table I). It did not recognize the 1934 sequence, which differs by an Asp to Glu substitution at position 372 and an Ala to Thr at position 373. F5 was tested on a set of truncated versions of the peptide with the 1968 sequence, each shorter than the other by one amino acid (13). The results gave a set of dose-response curves that showed that 366-379 was recognized ~10-fold more efficiently than 365-379, and 368-379 ~100-fold less efficiently than 365-379.

The same analysis has now been done with clone 11, which crossreacts with both the 1934 and 1968 sequences. The results are shown in Fig. 3. The most clear-cut difference in the set of dose-response curves is that, unlike clone F5, clone 11 recognizes 365-379 and 366-379 with equal efficiency.

To extend the analysis with clones 11 and F5, a set of amino acid substitutions was introduced into the 365-379 peptide of the 1968 sequence as summarized in Table III. To identify which amino acid was responsible for the ability of clone F5 to differentiate between the 1934 and 1968 sequences, the two independent substitutions Asp to Glu (372) and Ala to Thr (373) were introduced. Finally, the effect of a conservative substitution in the pair of hydrophobic

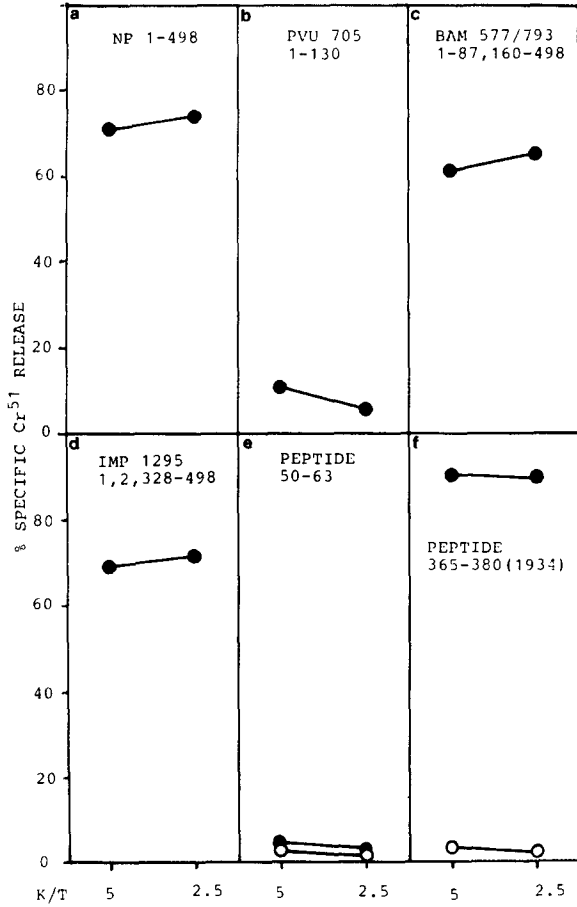


FIGURE 2. Recognition by C57BL/10 clone 11 of the same set of target cells as described in Fig. 1. (●) Clone 11 tested on transfected or peptide-pulsed target cells; (○) clone 11 tested on untreated L/D<sup>b</sup> cells.

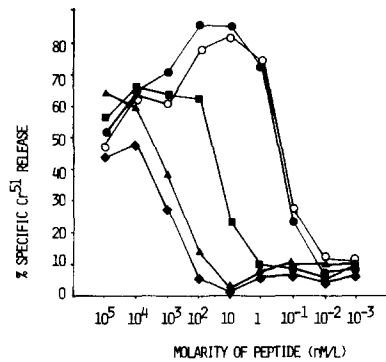


FIGURE 3. Recognition by clone 11 of LD<sup>b</sup> target cells in the presence of decreasing concentrations of truncated peptides all from the 1968 NP sequence. (●) 365-379; (○) 366-379; (■) 367-379; (▲) 368-379; and (◆) 369-379. Clone 11 was used at a K/T ratio of 2:1.

residues in the middle of the peptide was assessed by introducing Ile for Met at position 374.

The set of resulting dose-response curves for clones 11 and F5 are shown in Figs. 4 and 5, respectively. Comparison of Figs. 3 and 4 shows that the dose-response curve for clone 11 on the reference peptide 365-379 (1968) has shifted. In Fig. 3 the plateau level of lysis was higher (70-80%) and was reached at ~1

TABLE III  
Summary of the Effects on Recognition by Clones 11 and F5 of Amino Substitutions and Deletions in Peptide 365-379 (1986 Sequence)

Peptide	Wild-type 1,986 sequence	Relative potency of peptide compared with wild-type 365-379 (1986 sequence)	
		Clone 11	Clone F5
	I A S N E N M D A M E S S T L		
365-380; 1934 sequence	----- E T -----	~1	NR*
365-379 (D-E at 372)	----- E -----	~1/8	NR
365-379 (A-T at 373)	----- T -----	~1/8	~40
365-379 (M-I at 374)	----- I -----	~1/16	~50
366-379; 1968 sequence	-----	~1	~10

The effects are referred to in terms of the relative potency in the cytotoxicity assay of the substituted peptides compared with wild-type sequence as described in the text.

\* NR, Not recognized.

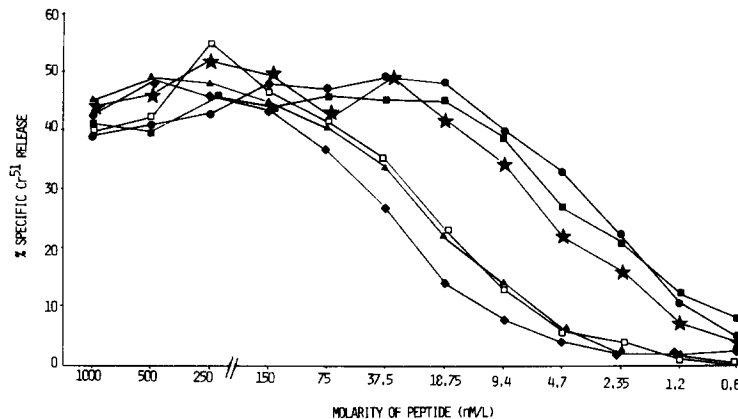


FIGURE 4. Recognition by clone 11 of LD<sup>b</sup> target cells in the presence of decreasing concentrations of peptides containing amino acid substitutions. (●) 365-379 (wild-type 1968); (■) 366-379 (wild-type 1968); (★) 365-380 (wild type 1934); (□) 365-379 (1968) with Asp to Glu substitution at position 372; (▲) 365-379 (1968) with Ala to Thr substitution at position 373; (◆) 365-379 (1968) with Met to Ile substitution at position 374. Clone 11 was used at a K/T ratio of 2.5:1.

nM/liter of peptide, whereas in Fig. 4 the plateau was lower (40-50% lysis) and was reached at ~10 nM/liter of peptide. This reduction in lytic activity of clone 11 correlated with recent (3 wk) thawing from frozen stock. Although the absolute levels of lysis by clone 11 varied in this way, the pattern of recognition on the various peptides remained constant (data not shown). In each experiment the reference peptide 365-379 (1968) was therefore included as an internal standard against which the relative potency of peptides with amino acid substitutions could be measured.

Fig. 4 shows that 365-379, 366-379 of the 1968 sequence and 365-380 of 1934 sequence were recognized with approximately equal efficiency by clone



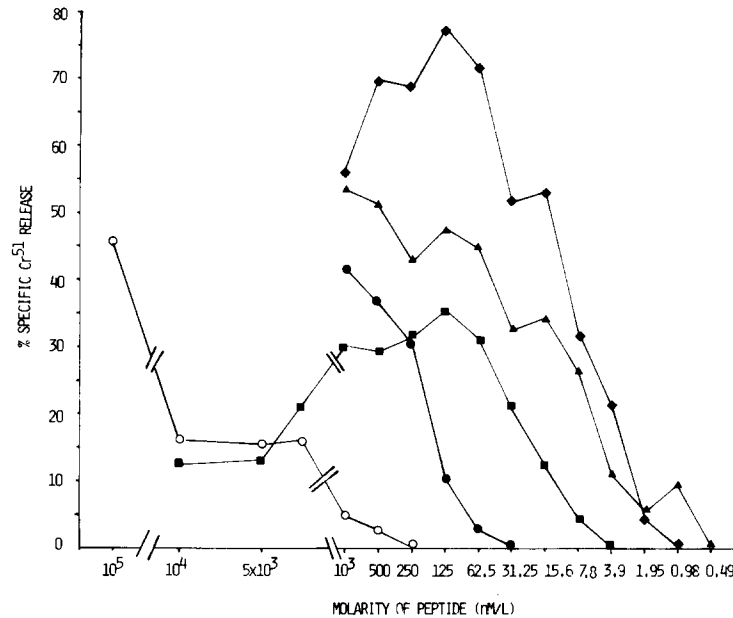


FIGURE 5. Recognition by clone F5 of LD<sup>b</sup> target cells in the presence of decreasing concentrations of peptides containing amino acid substitutions. (●) 365-379 (wild-type 1968); (■) 366-379 (wild-type 1968); (○) 368-379 (wild-type 1968); (▲) 365-379 with the Ala to Thr substitution at position 372; (◆) 365-379 with the Met to Ile substitution at position 374. Both the 365-380 (wild-type 1934) and 365-379 with the Asp to Glu substitution at position 372 failed to be recognized by F5 (data not shown). Clone F5 was used at a K/T ratio of 2:1.

11. The two peptides with single substitutions at positions 372 and 373 were recognized approximately eightfold less efficiently than the reference peptide 365-379. Thus, the two natural sequences containing either Asp(372), Ala(373) or Glu(372), Thr(373) were recognized more efficiently by clone 11 than peptides containing either one of the two alternative sequences. The peptide containing the substitution Met to Ile at position 374 was recognized ~16-fold less efficiently than the reference peptide.

Clone F5 gave a different set of dose-response curves with the same set of peptides, shown in Fig. 5. As mentioned above, both clones were restricted through the class I molecule Db. Neither the peptide 365-380 (1934 sequence) nor the peptide with the single substitution Asp to Glu at position 372 were recognized by clone F5 at any concentration tested (data not shown). This demonstrated that Asp at residue 372 controlled the specificity defined by clone F5. Of interest, and in contrast to clone 11, the single substitution Ala to Thr at position 373 was recognized ~40-fold more efficiently than the wild-type sequence. In addition, the Met to Ile substitution at position 374 also resulted in an ~50-fold increase in potency for clone F5. These differential effects on the recognition by the two clones are summarized in Table III.

*Comparison of the Recognition by CTL of Purified Recombinant NP and Peptide 366-379 in the Cytotoxicity Assay.* The NP from A/NT/60/68 influenza virus has been produced as a recombinant protein in *E. coli* (19). The NP gene from the same virus has been used throughout in the transfection experiments that

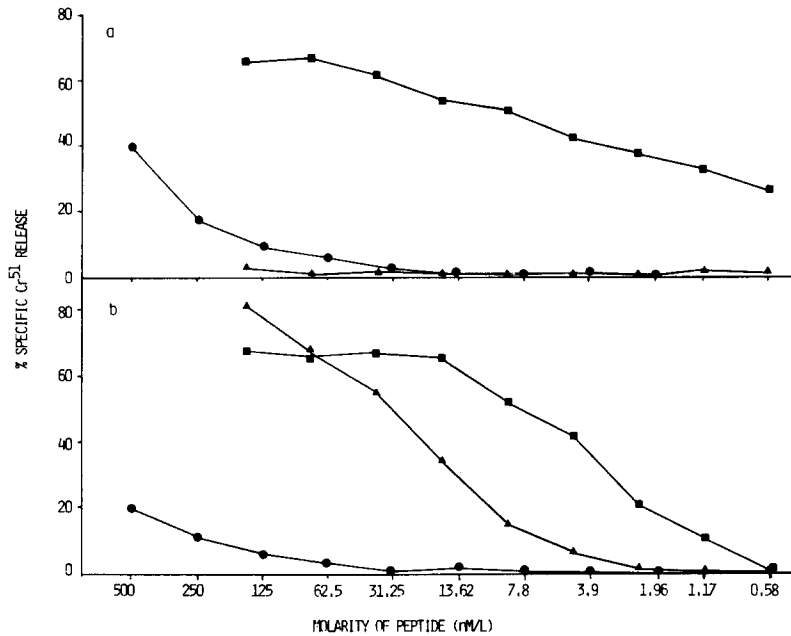


FIGURE 6. Recognition by (a) clone F5 and (b) clone 11 of LD<sup>b</sup> target cells in the presence of decreasing concentrations of peptides and recombinant NP. (■) 366–379 (wild-type 1968); (▲) 365–379 (1968) with the Asp to Glu substitution at position 372; (●) recombinant NP. The clones were used at a K/T ratio of 3:1.

defined the specificities of clones 11 and F5 (5, 10). In view of the fact that NP has been detected at the surface of influenza virus–infected cells (29, 30), but not on cells transfected with the NP gene (10), it was of interest to compare purified whole NP with appropriate peptides for its ability to sensitize target cells for lysis in the <sup>51</sup>Cr-release assay. Purified NP was titrated in the assay as described in Materials and Methods. The preparation used was judged to be predominantly correctly folded, as it reacted correctly with a panel of mAbs and bound viral RNA *in vitro* to form characteristic aggregates observed by electron microscopy (19).

Fig. 6 shows that at high concentration (0.5  $\mu$ M) whole NP was recognized inefficiently by clones 11 and F5. Higher concentrations could not be accurately used as the protein has a low solubility. Over the same molar concentration range the peptide 366–379 was found to be  $\sim$ 200-fold more efficient at inducing lysis of target cells by both CTL clones, i.e., an  $\sim$ 200-fold higher concentration of whole NP was necessary to induce the same level of target cell lysis. The peptide with the Asp to Glu substitution at position 372 was included in the experiment to highlight the differences in specificity between the two clones.

For some proteins, denaturation without degradation can be sufficient to allow recognition by class II–restricted T cells (31, 32). Recombinant NP was treated with 7 M guanidine HCl and was reduced and alkylated before testing in the cytotoxicity assay with clones 11 and F5. This treatment did not enhance the ability of the material to sensitize target cells. In two experiments the activity of the preparations was slightly reduced (data not shown).

### Discussion

The characteristic feature of the CTL response to influenza is the predominant specificity for conserved epitopes of the nonglycoproteins of the virus (1, 2, 5–9). This contrasts with the neutralizing antibody response that is predominantly directed at strain-specific epitopes on the viral glycoproteins (33). Evidence has accumulated that influenza-specific CTL are able to recognize proteins of the virus that do not have a known means of transport to the cell surface (5–9). In both mice and humans the viral nucleoprotein is an antigen recognized by CTL that crossreact between different A virus strains (5–7).

Table II and Figs. 1 and 2 show that the conserved epitopes on NP recognized by CBA and C57BL CTL can be defined with short peptides. These results complete our earlier analysis (10) with target cells transfected with deletion mutants of the NP gene. All four epitopes mapped in the deletion analysis have now been defined with synthetic peptides. A fifth epitope has been demonstrated (12, 13) with an additional peptide in the human CTL response. These results are summarized in Table I. A sixth peptide epitope recognized by BALB/c CTL (Kd-restricted) has recently been defined using the same techniques (Askonas, B. A., personal communication).

The finding that CTL can recognize short synthetic peptides *in vitro* raises the possibility of using peptide vaccines to prime or restimulate CTL *in vivo*. However, the strict genetic control of responsiveness to individual epitopes by the polymorphic class I MHC genes (11, 12) may limit the usefulness of this approach in the genetically diverse human population.

The data in Figs. 3–6 and summarized in Table III define the specificity differences between clones 11 and F5, both of which recognize the wild-type sequence 365–379 (1968 isolate) and both of which are restricted through the class I molecule Db. Certain points are worth considering. The major amino acid determining the specificity of clone F5 is the Asp to Glu substitution at position 372. The 1968 sequence with this change alone results in an approximately eightfold reduction in efficiency of recognition by clone 11, and abolishes recognition by clone F5. This demonstrates that clone F5 is sensitive to a conservative amino acid change, the addition of a single methylene group to the peptide being sufficient to completely prevent recognition.

Although reduced in potency, the peptide with Glu at position 372 was still recognized by clone 11. If a single site of interaction with Db exists for this peptide, the Asp to Glu change could not be acting by preventing association with the Db molecule, and the result obtained would imply that Asp at position 372 interacts with the F5 T cell receptor. The alternative possibility is that the peptide 365–379 interacts with two different sites on Db, recognized mutually exclusively by clones 11 and F5. In this case the Asp to Glu substitution could be thought of as preventing association of the peptide at one site on Db but not at the other. Competition experiments with the various peptides for binding to the isolated Db molecule may shed light on this point.

Another feature of interest is that certain amino acid substitutions in the peptide can have opposite effects on recognition by the two T cell clones (shown in Fig. 5 and summarized in Table III). For instance, the substitution of Ile for Met at position 374 resulted in an ~50-fold enhancement in potency of the

peptide for clone F5 and an ~16-fold reduction in potency for clone 11, when compared with the wild-type sequence. The molecular interpretations of this result again depend on whether more than one site of interaction with the Db molecule exists. If there is a single site, then any substitution interfering with the association of peptide with Db would be expected to reduce the efficiency of recognition by all Db-restricted CTL specific for that peptide, regardless of their fine specificities. The observed effects would then be interpreted by Ala (373) and Met (374) interacting slightly differently with the clone 11 and F5 T cell receptors, but not playing a role in the association with Db. An additional example of two class I-restricted epitopes on one 16-amino-acid peptide has recently been described in the Kd-restricted murine CTL response to the human HLA CW3 molecule (34), and is a common finding with class II-restricted T cells (35-39).

These results with recognition of peptides by class I-restricted T cells are closely related to those with class II-restricted T cells (reviewed in references 40-42) and are open to parallel interpretations. The correct interpretation of the results will depend on the structural analysis of the molecules involved and their interactions. The techniques described here can now be applied to the viral antigens recognized in association with the HLA molecules for which crystal structure is being elucidated.

Much recent discussion has concentrated on the question of whether degradation of protein antigens is an obligatory requirement for recognition by all T cells (42, 43). Examples exist of class II-restricted T cell clones that appear to recognize complete folded protein antigens (42), clones that recognize proteins that are denatured but not degraded, and finally, those that recognize short peptides but not complete proteins whether denatured or not (31, 32).

Influenza NP can be detected with antibody at the surface of influenza virus-infected cells (10, 29, 30). This is surprising because its sequence contains neither a hydrophobic signal peptide, nor a characteristic membrane anchor sequence. In view of its presence on the infected cell surface the question arises whether the epitopes we have defined *in vitro* with short peptides could be recognized *in vivo* as part of the complete protein.

Fig. 6 shows a comparison of recognition by clones 11 and F5 of the complete recombinant NP with the peptide 366-379. The dose-response curves show that the peptide is recognized ~200-fold more efficiently than the whole protein by these T cell clones in this type of assay. These results are consistent with the work of Wraith and Vessey (44) who found that the Db-restricted CTL clone A3.1 (which is specific for the same region of sequence as clone F5, but in the 1934 NP) (13), could proliferate in response to high concentrations of the 1934 NP (0.45-1.8  $\mu$ M/liter) presented by a fixed or ammonium chloride treated macrophage preparation.

The low level of recognition of whole NP compared with peptide in the 6-h cytotoxicity assay suggests that the epitopes around the sequence 366-379 are poorly exposed on the complete recombinant molecule in solution. The denaturing conditions tested had no detectable effect on revealing these epitopes to clones 11 or F5. These results extend the work on transfected target cells (5, 10)

and are consistent with the suggestion that degradation of NP occurs in the virus-infected cell before recognition by CTL.

The L cells used as targets might be expected to be capable of endocytosing soluble NP and degrading it within membrane-bound vesicles (45), as is thought to occur during presentation with class II molecules (46). However, if this mechanism is at work it appears to be inefficient for the epitope studied here under these conditions. This result is related to the findings of Morrison et al. (47), who have shown that class I-restricted CTL specific for hemagglutinin were not able to recognize A20-1.11 B lymphoma cells exposed to the soluble protein in conditions where presentation to class II-restricted T cells occurred efficiently. Taken with the finding that HA-specific CTL can recognize target cells that express the HA molecule as an obligatory cytoplasmic protein (48), these results imply that the mechanisms by which protein antigens are made visible to class I- and class II-restricted T cells may be different.

### Summary

The conserved epitopes of influenza nucleoprotein (NP) recognized by class I MHC-restricted CTL from CBA (H-2k) and C57BL/10 (H-2b) mice have been defined in vitro with synthetic peptides 50-63 and 365-379, respectively. Two D<sup>b</sup>-restricted clones were described that recognize different epitopes on peptide 365-379. Finally, the recognition of complete NP was shown to be ~200-fold less efficient than peptide in the cytotoxicity assay. These phenomena are closely related to results with class II-restricted T cells and they strengthen the hypothesis that influenza proteins are degraded in the infected cell before recognition by class I-restricted CTL.

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