*Immunology* 1989 **66** 163–169

# Influenza-specific cytotoxic T-cell recognition is inhibited by peptides unrelated in both sequence and MHC restriction

H. C. BODMER,* J. M. BASTIN,† B. A. ASKONAS* & A. R. M. TOWNSEND† **Immunology Division, National Institute for Medical Research, Mill Hill, London and †Institute for Molecular Medicine, John Radcliffe Hospital, Oxford*

*Accepted for publication 3 October 1988*

## SUMMARY

Two related peptides from the nucleoprotein (NP) sequence 365–380, derived from influenza virus isolates A/PR/8/34 and A/NT/60/68, are recognized by mutually exclusive sets of $D^b$ (Class I)-restricted cytotoxic T-lymphocyte (CTL) clones. These peptides compete with each other for presentation on $D^b$-bearing target cells *in vitro*. A $K^k$-restricted nucleoprotein epitope (NP 50–63), which is unrelated in sequence, competes more efficiently on H-$2^b$ target cells but is not itself recognized by virus-specific CTL from influenza-infected H-$2^b$ mice. A peptide sequence from the class I molecules Cw3 and $D^b$ can also compete, but additional unrelated peptides do not do so at equimolar concentrations. Our results show that competition is at the level of the target cell and imply that the binding specificity of the class I molecule $D^b$ is broader than indicated by the immune response phenotype of the C57BL (H-$2^b$) mouse.

## INTRODUCTION

The currently accepted model of recognition by T cells proposes that a major histocompatibility molecule binds a fragment of antigen and this complex is recognized by the T-cell receptor. It is likely, therefore, that each MHC molecule would be able to bind a large number of different peptides in order to accommodate the wide range of an individual's T-cell responses. The model is supported by the findings of a number of workers who have correlated binding of peptides to class II molecules with specific T-cell responses (Babbitt *et al.*, 1985; Watts & McConnell, 1986; Buus *et al.*, 1986, 1987; Guillet *et al.*, 1986, 1987; Lakey *et al.*, 1987a; Allen *et al.*, 1987; reviewed by Townsend & McMichael 1987).

The crystal structure of the class I molecule HLA A2 has revealed a putative binding site for peptide antigens. (Bjorkman *et al.*, 1987a, b). However, measurement of direct binding of peptides to class I molecules has not been reported.

In this paper we have used murine cytotoxic T-cell (CTL) recognition of influenza A virus nucleoprotein (NP) to study competition between peptides for presentation to class I MHC-restricted T cells. Four distinct epitopes of NP have previously been defined for murine or human CTL: residues 50–63 recognized in the context of $K^k$, residues 147–158 in the context of $K^d$, 335–349 in the context of HLA B37 and residues 365–379 in the context of $D^b$ (Townsend *et al.*, 1986; Bastin *et al.*, 1987; Taylor *et al.*, 1987; Bodmer *et al.*, 1988).

Correspondence: Dr H. C. Bodmer, Institute for Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DU, UK.

In general there is little variation of the NP sequence between influenza A virus strains; however, in the $D^b$-restricted NP epitope there are two amino acid differences between the sequences from 1934 (A/PR/8/34) and 1968 (A/NT/60/68) virus isolates (Winter & Fields 1981; Huddleston & Brownlee, 1982). $D^b$-restricted T-cell clones (Table 1) are either specific for one or other sequence or cross-reactive, (Townsend & Skehel, 1984; Townsend *et al.*, 1986, Bastin *et al.*, 1987). We show here that competition can occur between related peptides from this region of NP, leading to inhibition of target cell lysis. We also show that certain peptides of unrelated sequence and restricted by different class I MHC molecules can compete, and that this property of competition *in vitro* does not correlate with the induction of a $D^b$-restricted CTL response *in vivo*. Competition between peptides during pretreatment of target cells indicates that the inhibition is at the level of the target cell, and is likely to be for binding to the class I restriction element.

## MATERIALS AND METHODS

*Mice*

C57BL/10, C57BL/6, CBA and (C57.BL/10 × $DBA_2$) $F_1$ mice were bred under specific pathogen-free conditions at NIMR.

*Influenza A virus strains*

The recombinant influenza A viruses A/X31 and E61-13-H17 differ in the origin of their genes for nucleoprotein (Baez, Palese & Kilbourne, 1980). The X31 NP gene is derived from A/PR/8/34, the E61-13-H17 NP gene from A/HK/8/68 (Lubeck, Palese & Schulman, 1983; where E61-13-H17 is referred to as recombi-

nant 33). viruses were grown in the allantoic cavity of 11-day embryonated chick eggs. Infectious allantoic fluid was stored at −70°.

*Polyclonal cytotoxic T-cell culture*

(C57BL × $DBA_2$) $F_1$ mice were primed by intranasal infection with four haemagglutination units (HAU) of A/X31. Cultures were set up as previously described (Lin & Askonas, 1981). Briefly spleen cells, 3–12 weeks after priming, were stimulated *in vitro* with A/X31-infected syngeneic spleen cells from normal mice. Cultures were incubated in RPMI-1640 + 10% fetal calf serum (FCS) for 5 days at 37°, 6% $CO_2$.

*Cytotoxic T-cell clones*

F5 is a $D^b$-restricted C57BL/6 CTL clone specific for the 1968 NP sequence 365–379 (Townsend *et al.*, 1984; Bastin *et al.*, 1987).

Clone 11 is a $D^b$-restricted C57BL/10 CTL clone specific for NP 365–380 and cross-reactive for 1934 and 1968 sequences (Bastin *et al.*, 1987).

B4 is a $D^b$-restricted C57BL/6 clone specific for 1934 NP 365–380 (Townsend *et al.*, 1984, 1986; Wraith & Vessey, 1986).

*Polyclonal CBA (H-$2^k$) CTL specific for NP 50–63*

These were derived from male or female CBA mice primed for 2 weeks by either i.v. injection of 100 HAU of A/PR/8/34 virus or by intra-nasal infection with five HAU of E61-13-H17 virus. Primed spleen cells ($1·5 \times 10^7$) were stimulated with $5 \times 10^6$ peptide-treated spleen cells for 5 days at 37°, 5% $CO_2$. Stimulator spleen cells from normal animals were irradiated with 2000 rads, and resuspended in 0·5–1 ml medium containing NP 50–63 at $1–5 \times 10^{-5}$ M/l for 1 hr at 37°. They were then washed in phosphate-buffered saline (PBS) before addition to responder cells. The CTL generated showed peptide- and virus-specific cytotoxic activity, and were maintained by weekly stimulation with peptide pulsed feeder cells and recombinant human IL-2 (10 Cetus U/ml, Cetus Corp, Emeryville, CA) (Bastin *et al.*, 1987).

*Peptides*

Peptides were synthesized as described previously (Townsend *et al.*, 1986). CW3 171–182 was donated by Dr Maryanski and all others were provided by Dr J. Rothbard.

*Target cells*

The following cells were used: (i) L cells (H-$2^k$) transfected with the gene for $D^b$ as previously described (L/$D^b$) (Townsend *et al.*, 1984; (ii) EL-4 cells (H-$2^b$); (iii) C57BL/10 peritoneal exudate cells (H-$2^b$) induced by i.p. injection of 1·5 ml 3% w/v fluid thioglycollate (Difco, Detroit, MI) for 3–6 days (Townsend *et al.*, 1983).

*$^{51}$Cr-release cytotoxicity assay*

A standard procedure was used as described previously (Zweerink *et al.*, 1977; Townsend *et al.*, 1986; Bodmer *et al.*, 1988). $^{51}$Cr-labelled target cells were either untreated or treated with peptides at varying concentrations for 90 min and then washed three times before addition to the assay ($1–2 \times 10^4$/well). CTL were diluted and added at the appropriate K:T ratio as indicated (Fig. 6c). In the indicated experiments CTL were incubated with peptide for 90 min and washed three times before addition to the assay.

% target lysis =

$$\frac{\text{c.p.m. sample release} - \text{c.p.m. spontaneous release}}{\text{c.p.m total release} - \text{c.p.m. spontaneous release}} \times 100.$$

Spontaneous $^{51}$Cr release of target cells in the absence of CTL was always < 18% of the total $^{51}$Cr released by 2·5% Triton X-100.

## RESULTS

### Both related and unrelated peptides can inhibit CTL-mediated target cell lysis

T-cell clones derived from virus-primed C57BL/6 or /10 mice (H-$2^b$) are either cross-reactive for the region 365–380 of NP (clone 11), or specific for either the 1968 (clone F5) or the 1934 sequence (clone B4) (Townsend *et al.*, 1986; Table 1), which differ as residues 372 and 373 (Table 2).

Figure 1 demonstrates lysis of $^{51}$Cr-labelled EL-4 (H-$2^b$) target cells by clones F5 and B4 in the presence of their respective target peptides NP 366–379 (1968) and NP 365–380 (1934), and the effect of adding various competing peptides at molar excess. The dose-response curves of the two target peptides with their respective T-cell clones are similar. When $10^{-5}$ M NP 365–380 (1934) are added to the assay with the target peptide NP 366–379 (1968) (a), target cell lysis by clone F5 is inhibited when the competitor is present at 100- or 1000-fold excess. The same is true for clone B4 (b) when excess competitor peptide NP 366–379 (1968) is added to the assay. Inhibition is overcome by increasing the concentration of the target peptide to a point where the molar ratio of inhibitor to target peptide is < 10:1.

Competition is not limited to related peptides, which are themselves $D^b$-restricted epitopes. An excess of NP 50–63, an

**Table 1.** CTL clones, lines and cultures used in this report

| Name | Mouse strain | H-2 restriction | NP epitope | Reference |
|---|---|---|---|---|
| B4 | C57BL/10 | $D^b$ | 365–380 (1934) | Wratih & Vessey (1986)<br>Townsend *et al.* (1986) |
| F5 | C57BL/6 | $D^b$ | 365–379 (1968) | Townsend *et al.* (1986) |
| Clone 11 | C57BL/10 | $D^b$ | 365–380 cross-reactive | Bastin *et al.* (1987) |
| CBA polyclonal | CBA | $K^k$ | 50–63 | Bastin *et al.* (1987) |
| $F_1$ polyclonal | (C57BL × $DBA_2$) $F_1$ | H-$2^b$ | 365–380 | Taylor *et al.* (1987) |
| | | H-$2^d$ | 147–158 | |

**Table 2.** Peptides of unrelated sequence compete for presentation to clone F5

| Target peptide | | | Class I restriction |
|---|---|---|---|
| NP 365–379 | 1968 | I A S N E N M D A M E S S T L | $D^b$ |
| Competitors | | | |
| NP 365–380 | 1934 | - - - - - - E T - - - - - - - | $D^b$ |
| | E372 | - - - - - - - E - - - - - - - | $D^b$ |
| NP 50–63 | | - D Y E G R L I Q N - L T I | $K^k$ |
| | D55 | - D Y E G - L I Q N - L T I | $K^k$ |
| | E58 | - D Y E G R L I - N - L T I | $K^k$ |
| HLA Cw3 171–182 | | Y L K - G K E T L Q R A | $K^d$ |
| H-2 Db 171–182 | | Y L K - G N A T L L R T | — |
| Non-competitors | | | |
| NP 147–158 | | T Y Q R T R - L V R T G | $K^d$ |
| R156 MINUS | | T Y Q R T R - L V T G | $K^d$ |
| NP 335–349 | | - A A F E - L R V L - F I R G | HLA-B37 |
| RSV F 71–86 | | G T D A K V K L I K Q - L D K Y | — |
| 'NP 366–379 | | COOH - - - - - - - - - - - - - - - NHH D-Isomer | — |

Summary table of peptides which compete for presentation with NP 365–379 to CTL clone F5. Peptides are arranged to show maximum sequence identity to NP 365–379 (1968), a dash represents amino acid identity in that position with the target peptide.

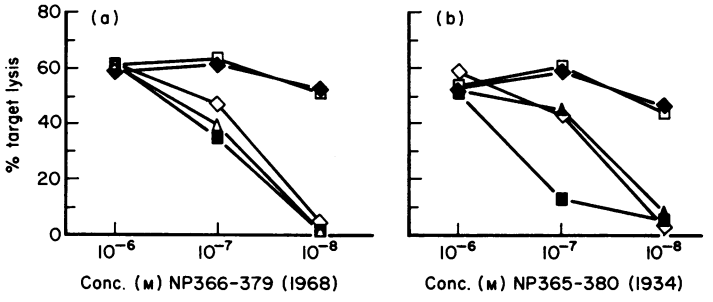


**Figure 1.** Competition between both related and unrelated peptides leading to inhibition of target cell lysis by two non-cross-reactive NP-specific CTL clones. The target peptides are titrated against a constant concentration ($10^{-5}$ M) of competitor peptide. Target cells were EL-4 cells labelled with $^{51}Cr$. Competing and target peptides were mixed and added to the 4 hr assay. The K:T ratio = 2. (a) CTL clone F5, target peptide NP 366–379 (1968). (b) CTL clone B4, target peptide NP 365–380 (1934). Competing peptides: (□) no competitor peptide; (◆) NP 147–158; (■) NP 50–63; (◇) Cw3 171–182; (△) NP 365–380 (1934); (▲) NP 365-379 (1968). Non-specific lysis and lysis in the presence of competitors alone < 7%.

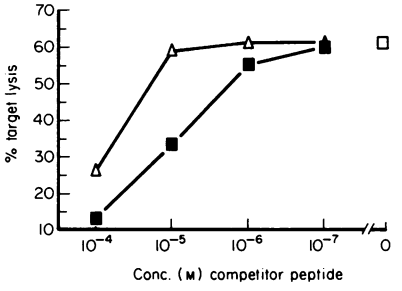


**Figure 2.** Titration of competitor peptide against a constant concentration of the target peptide on lysis of L/$D^b$ target cells by CTL clone F5 at K:T = 3. Target peptide was NP 365–379 2 × $10^{-7}$ M present throughout the assay with competing peptides NP 50–63 [■] or NP 365–380 (1934) [△]. No competition [□]. Non-specific lysis or lysis of L/$D^b$ cells in the presence of competitor peptides alone < 8%.

epitope for $K^k$-restricted NP-specific CTL (Bastin *et al.*, 1987), is a more efficient inhibitor of lysis than either of the $D^b$-restricted peptides, most noticeable in the case of CTL clone B4 (Fig. 1). Peptides NP 147–158 and Cw3 171–182 (Table 2) are both epitopes recognized by $K^d$-restricted CTL (Bodmer *et al.*, 1988; Maryanski *et al.*, 1988) and they compete with each other for presentation with $K^d$ (Pala *et al.*, 1988). While NP 147–158 has no effect on target cell lysis by either $D^b$-restricted CTL clone (and serves as a negative control), Cw3 171–182 is as efficient a competitor as either of the $D^b$-restricted peptides.

### Inhibition is dose-dependent and relates to the ratio of competitor to target peptides

In the experiments in Fig. 1 a constant concentration of competing peptide was used in the assay with titration of the target peptide. Figure 2 shows the effect of titrating the competing peptides at a constant high concentration of the target peptide, NP 365–379 (1968) 2 × $10^{-7}$ M, with CTL clone F5. The target cells in this experiment were L cells transfected with the gene $D^b$ (L/$D^b$). NP 50–63 is again shown to be a more efficient competitor than NP 365–380 (1934). Competition occurred at a molar ratio of inhibitor to target peptide of between 50 and 500:1 (50–63) and 500:1 (365–380'34); these are similar ratios to Fig. 1.

### Similar patterns of peptide competition are seen with $F_1$ polyclonal CTL and peritoneal exudate cells as targets

Polyclonal CTL from H-$2^{b\times d}$ $F_1$ mice, primed by influenza infection, are able to recognize epitopes of both parental strains

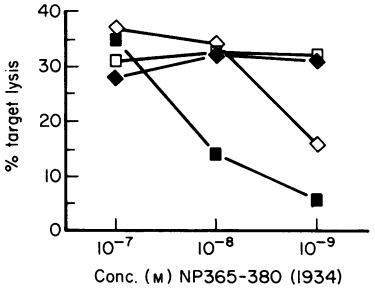


**Figure 3.** $F_1$ polyclonal CTL are inhibited in a pattern similar to the $D^b$ restricted clones. Polyclonal (C57BL/10 × $DBA_2$) $F_1$ (CTL) were used at K:T = 8. Target cells were C57BL/10 ($H\text{-}2^b$) peritoneal exudate cells, 2 × $10^4$/well. Peptides were added directly to the assay. The target peptide NP 365–380 was at the concentrations shown on the axis. Competitor peptides were added at $10^{-5}$ M; NP 147–158 [◆], NP 50–63 [■], CW3 171–182 [◇] or no competitor [□]. Non-specific lysis or lysis in the presence of competitor peptides alone < 8%.

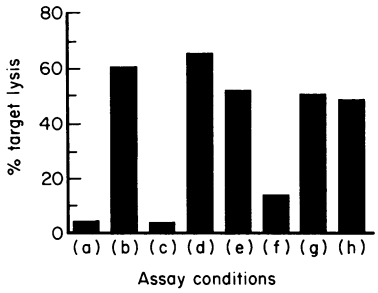


**Figure 4.** Competition only occurs when target cells are exposed to competitor and target peptides simultaneously. CTL clone 11 (see Table 1) K:T = 3. $D^b$ target cells, $10^4$/round-bottomed well. (a) lysis of untreated L/$D^b$. (b) target peptide NP 365–379 (1968) 2 × $10^{-8}$ M alone in assay; (c) a mixture of NP 365–379 (1968) 2 × $10^{-8}$ M and NP 50–63 $10^{-4}$ M in assay; (d) a mixture of NP 365–379 (1968) 2 × $10^{-8}$ M and RSV F 71–86 $10^{-4}$ M in assay; (e) L/$D^b$ cells pretreated with NP 365–379 (1968) 2 × $10^{-8}$ M 90 mins and washed before addition to assay; (f) L/$D^b$ cells pretreated with a mixture of NP 365–379 (1968) 2 × $10^{-8}$ M and NP 50–63 $10^{-4}$ M; (g) L/$D^b$ cells pretreated with NP 365–379 (1968) 2 × $10^{-8}$ M and RSV F 71–86 $10^{-4}$ M; (h) L/$D^b$ cells pretreated with NP 365–379 (1968) 2 × $10^{-8}$ M; NP 50–63 $10^{-4}$ M added to assay.

**Table 3.** CBA ($H\text{-}2^b$) polyclonal CTL can be inhibited by peptide competition

| Target ($10^{-7}$ M) lysis | Competitor ($2{\cdot}5 \times 10^{-4}$ M) | % target cell |
|---|---|---|
| No peptides | | 0 |
| NP 50–63 | — | 34 |
| NP 50–63 | NP 50–63 D55 | 15 |
| NP 50–63 | NP 365–379 | 2 |

CBA polyclonal CTL at K:T = 10 with L/$D^b$ target cells 2 × $10^4$/well in a $^{51}Cr$-release assay.

when presented on a target cell expressing the appropriate class I MHC molecules (Taylor *et al.*, 1987). A polyclonal CTL culture from A/X31-primed (C57BL × $DBA_2$) $F_1$ mice recognized NP 365–380 (1984) on C57BL ($H\text{-}2^b$) peritoneal exudate target cells. As with the $D^b$-restricted CTL clones, NP 50–63 and Cw3 171–182 inhibit target cell lysis while NP 147–158 does not (Fig. 3). The same CTL lyse $H\text{-}2^d$ target cells treated with NP 147–158 but not NP 365–380 (not shown), and the pattern of inhibition seen was similar to that obtained with the NP-specific $K^d$-restricted CTL clones (Pala *et al.*, 1988). These patterns of inhibition therefore relate to the antigen specificity and MHC restriction of both cloned and polyclonal T cells, and do not vary with the source of the target cell.

### Inhibition of $K^k$-restricted CTL lysis by related and unrelated peptides

NP 50–63 has been shown previously to be an epitope for $K^k$-restricted CTL (Bastin *et al.*, 1987). An analogue of NP 50–63 with an aspartate for arginine substitution at position 55 (Table 2) is not recognized by CBA polyclonal CTL (not shown) but can compete with wild-type peptide to produce 50% inhibition of target cell lysis at a molar excess of 2500 (Table 3). The unrelated peptide NP 365–379 (1968) also competes at this molar excess but leading to complete inhibition of lysis. Although concentrations of competitors required to achieve inhibition in this system are high (250 × $10^{-4}$ M), these levels are not toxic in themselves as increasing the concentration of the target peptide NP 50–63 can overcome the inhibition (not shown).

### Competition between peptides requires simultaneous exposure to target cells

Figure 4 demonstrates inhibition of L/$D^b$ target cell lysis by the NP-cross-reactive CTL clone 11. When both the target peptide, NP 365–379 (1968), and the competing peptide NP 50–63 (5000:1 ratio) are present in the assay there is almost complete inhibition of lysis (as previously shown in Fig. 1 with clones B4 and F5). Peptide F 71–85 from respiratory syncytial virus fusion protein (not so far defined as a T-cell epitope) serves as a negative control. If the target cells are pretreated with a mixture of NP 366–379 and NP 50–63 for 90 min and then washed before addition to the assay, inhibition of lysis is again seen (Fig. 4 e and f). However, if the target cell is pretreated with the target peptide alone, and the competing peptide is added to the assay (Fig. 4h) no inhibition results. In this assay the competing peptide NP 50–63 or the control (RSVF 71-86) at $10^{-4}$ M and the target peptide NP 365–379 (1968) at 2 × $10^{-8}$ M were present throughout the assay, giving 5000:1 ratio of inhibitor to target peptide. These results show that once a target cell was pretreated with the target peptide, it was no longer possible to inhibit its lysis with a competitor within the time period of the assay. They also imply that competition in these experiments was occurring at the level of the target cell.

Figure 5 demonstrates that pretreatment of target cells of CTL with competitor peptides alone (at 3 × $10^{-5}$ M), before the addition of target peptide to the assay, also does not lead to inhibition of target cell lysis. Pretreatment of the target cell or the CTL with up to $10^{-4}$ M competing peptide did not produce inhibition of target cell lysis (not shown). In summary these results show that for competition to occur cells must be exposed to the recognized peptide and its competitor simultaneously.

Finally, we attempted to use the most efficient competitor peptide (NP 50–63) to block the presentation of the 365–379

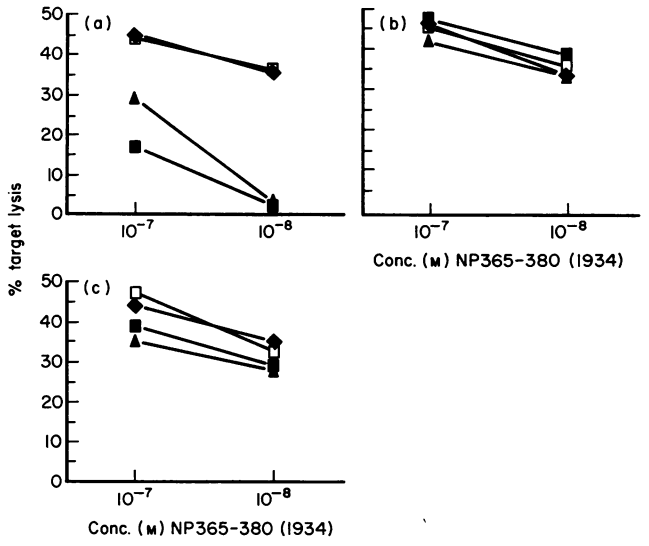


**Figure 5.** Pretreatment of target cells or CTL with competing peptide alone does not lead to inhibition. CTL clone B4, K:T = 4; EL-4 target cells. Competing peptides were used at $3 \times 10^{-5}$ M either during pretreatment or in the assay. Target peptide NP 365–380 (1934) was present in the assay at concentrations shown. (□) no competitor; (▲) NP 366–379 (1968); (■) NP 50–63; (◆) NP 147–158. (a) Both peptides, target and competitor, present in the assay. (b) Pretreatment of target cells with competing peptide for 90 min and washed three times before addition to assay. Target peptide in assay. (c) Pretreatment of CTL clone b4 with competing peptide for 90 min and washed. Target peptide in assay.

epitope of NP expressed on influenza virus-infected cells. Despite preincubating the target cells (H-$2^b$) with $2 \times 10^{-4}$ M NP 50–63 for 1 hr, and ensuring that the competitor was present throughout the virus infection and $^{51}$Cr-release assay at $10^{-4}$ M, no competition was detected (not shown).

Several additional peptides, including the retro-D isomer of NP 366–379, were found that neither stimulated nor competed for presentation to clone F5, as summarized in Table 2.

## DISCUSSION

Our experiments show that competition beween synthetic peptides can lead to inhibition of target cell lysis by influenza A virus NP-specific CTL. The finding (Fig. 1) that NP 365–380 (1934) competes with NP 366-379 (1968) and vice versa for presentation to appropriate $D^b$-restricted CTL clones supports our previous suggestion that the two residues that differ (Asp/Glu at position 372 and Ala/Thr at position 373) in these sequences are predominantly involved in contacts with T-cell receptors (Bastin *et al.*, 1987). The result is consistent with competition at a single peptide binding site on $D^b$, and is similar to findings by Maryanski *et al.* (1988) and Pala *et al.* (1988), who have demonstrated competition between closely related $K^d$-restricted peptides derived from the HLA-Cw3 and A24 molecule, and between $K^d$-restricted peptides of differing sequence.

We extend those results by finding competitors that were unrelated to the $D^b$-restricted peptide NP 365–380 in both sequence and MHC restriction (Table 2). The peptide 50–63 of NP, a $K^k$-restricted epitope, is a more effective competitor than either the 1968 or 1934 sequences of 365–380 for $D^b$-restricted recognition (Figs 1 and 2). Non-specific or toxic effects can be ruled out as NP 50–63 had no inhibitory effect on HLA-A2-restricted recognition of an inflenza matrix peptide by specific human CTL (Gotch *et al.*, 1987; F. Gotch, unpublished data), nor did it reduce lysis by clone 11 of target cells that had been pretreated with the target peptide NP 365–379 (Fig 4).

Both the competitors NP 50–63 and Cw3 171–182 are themselves target epitopes when presented to specific CTL in association with $K^k$ and $K^d$, respectively. However, no relationship between T-cell epitopes restricted by an identified class I MHC molecule and their ability to compete for $D^b$ presentation can be inferred, as neither an additional $K^d$-restricted peptide (NP 147–158) nor the HLA B37-restricted epitope NP 335-349 competed for presentation with $D^b$ (Table 2). In addition, the deletion of Arg 156 from NP 147–158, which enhances its potency by 1000-fold as an epitope and 100-fold as a competitor for peptides restricted through $K^d$ (Bodmer *et al.*, 1988; Pala *et al.*, 1988), does not enable it to compete for association with $D^b$.

Of the remaining negative controls in Table 2, the retro-D isomer of NP 366–379 is of interest, as it neither stimulated nor blocked clone F5 which is specific for the L-isomer. This result implies that reversing the peptide bonds in the sequence 366–379 abolishes it ability to associate with the peptide binding site on the $D^b$ molecule and extends work by Lamb *et al.* (1986) who found that the retro-D isomer of a haemaglutinin peptide was not recognized by class II-restricted T cells.

Since the $K^d$-restricted epitope of HLA Cw3, 171–182, was an effective competitor for $D^b$-restricted recognition, we synthesized and tested the homologous sequence from the $D^b$ molecule. The peptide $D^b$ 171–182 was as effective in competing with NP 366-379 as NP 50-63 (Table 3 and data not shown). This sequence forms the extreme (relatively) non-polymorphic C-terminal region of the $\alpha_2$ domain of $D^b$. The result indicates that peptides derived from the $D^b$ molecule itself can associate with the $D^b$ peptide-binding site, and is consistent with results showing that some A2-specific alloreactive CTL may recognize an HLA-A2-derived peptide in the A2 binding site (Parham *et al.*, 1987; Clayberger *et al.*, 1987).

In our experiments, inhibition only occurred when target and competitor peptides were simultaneously exposed to the target cell, either during pretreatment or continuously during the assay. Failure to inhibit recognition of a preformed target (i.e. either infected with influenza virus or pretreated with the target peptide alone) would be consistent with a slow off rate of target peptide bound to MHC, as has been found in studies of direct binding of peptides to class II molecules (e.g. Watts & McConnell, 1986; Buus *et al.*, 1987). Lack of inhibition by pretreatment with a competitor peptide may be due to failure to saturate all MHC sites (which are being continuously produced) with competitor. It is not known how many molecules of peptide a single CTL needs to recognize in order to trigger lysis of the target cell, although from the low concetrations of peptide required for maximum target cell sensitization, this number is likely to be small (Townsend, 1987; Bodmer *et al.*, 1988). For example, assuming a binding constant of $2 \times 10^{-6}$ M (Babbit *et al.*, 1985; Buus *et al.*, 1986) and approximately $2 \times 10^5$ class I molecules/cell, the number of bound peptides/target cell would be between 1 and 1000 in equilibrium with free peptide at $10^{-11}$–$10^{-8}$ M.

The majority of reports describing competition between

class II MHC-restricted peptides have noted that competitor peptides are usually related by also being epitopes for the particular restriction element (Buus *et al.*, 1987; Guillet *et al.*, 1987; Allen *et al.*, 1987). However, recently Lakey, Margoliash & Pierce (1987) have reported less specific competition in a class II-restricted system. They attribute this to competition for association with a non-MHC peptide-binding protein. The data reported in this paper are unlikely to be explained by competition between peptides for a non-polymorphic peptide binding protein because (C57BL/10 × $DBA_2$) $F_1$ H-$2^{b\times d}$ target cells present both NP 147–158 in association with $K^d$, and NP 366–379 in association with $D^b$, to mutually exclusive T-cell clones. Both peptides therefore bind the $F_1$ target cell surface, but NP 147-158 in excess does not inhibit the presentation of NP 366-379 either on an H-$2^b$ target (Figs 1 and 3) or on an $F_1$ H-$2^{b\times d}$ target (not shown).

Our results imply that NP 50–63 and NP 366–379 are both able to associate with $K^k$ and $D^b$ class I molecules *in vitro*. However, we have not been able to detect a CTL response to 50–63 with $D^b$, nor 365-379 with $K^k$, from mice infected with influenza virus. One possible explanation for this may be that in natural infection the fragments of NP generated during processing differ in length from the peptides used in these experiments. The natural fragment containing the sequence 50-63 may then bind preferentially to $K^k$. Alternatively receptors capable of recognizing 50-63 + $D^b$ may not exist in C57BL mice.

## ACKNOWLEDGMENTS

We wish to thank Dr J. Rothbard, Imperial Cancer Research Fund, London and Dr J. Maryanski, Ludwig Institute, Lausanne for kindly donating peptides, the Cetus corporation for providing recombinant human IL-2 and the MRC of Great Britain for support.

Dr Helen C. Bodmer is in receipt of a MRC Training Fellowship.

## REFERENCES

Allen P.M., Matsueda G.R., Evans R.J., Dunbar J.B., Marshall G.R. & Unanue E.R. (1987) Identification of the T-cell and Ia contact residues of a T-cell antigenic epitope. *Nature (Lond.)*, **327,** 713.

Babbit B., Allen P., Matsueda G., Haber E. & Unanue E. (1985) Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature (Lond.)*, 317, 359.

Baez M., Palese P. & Kilbourne E.D. (1980) gene composition of high yielding influenza vaccine strains obtained by recombination. *J. infect. Dis.* **141,** 362.

Bastin J., Rothbard J., Davey J., Jones I. & Townsend A. (1987) Use of synthetic peptides of influenza nucleoprotein to define epitopes recognized by class I restricted cytotoxic T lymphocytes. *J. exp. Med.* **165,** 1508.

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

Bjorkman P.J., Saper M.A., Samraoui B., Bennett W.S., Strominger J.L. & Wiley D.C. (1987b) The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature (Lond.)*, **329,** 512.

Bodmer H.C., Pemberton R.M., Rothbard J.B. & Askonas B.A. (1988) Enhanced recognition of a modified peptide antigen by cytotoxic T cells specific for influenza nucleoprotein. *Cell*, **52,** 253.

Buus S., Sette A., Colon S.M., Jenis D.M. & Grey H.M. (1986) Isolation and characterization of antigen-Ia complexes involved in T cell recognition. *Cell*, **47,** 1071.

Buus S., Sette A., Colon S.M., Miles C. & Grey H.M. (1987) The relationship between major histocompatibility complex (MHC) restriction and the capacity of Ia to bind immunogenic peptides. *Science*, **235,** 1353.

Clayberger C., Parham P., Rothbard J., Ludwig D.S., Schoolnik G.K. & Krensky A. (1987) HLA-A2 peptides can regulate cytolysis by human allogeneic T lymphocytes. *Nature (Lond.)*, **330,** 763.

Gotch F., Rothbard J., Howland K., Townsend A. & McMichael A. (1987) Cytotoxic T lymphocytes recognise a fragment of influenza virus matrix protein in association with HLA-A2. *Nature (Lond.)*, **326,** 881.

Guillet J., Lai M., Briner T.J., Buus S., Sette A., Grey H.M., Smith J.A. & Gefter M.L. (1987) Immunological self, nonself discrimination. *Science*, **235,** 865.

Guillet J.G., Lai M.Z., Briner T.J., Smith J.A. & Gefter M.L. (1986) Interaction of peptide antigens and class II major histocompatibility complex antigens. *Nature (Lond.)*, **324,** 260.

Huddleston J.A. & Brownlee G.G. (1982) The sequence of the nucleoprotein gene of human influenza A virus strain of A/NT/60/68. *Nucl. Acids. Res.* **10,** 1029.

Lakey E.K., Casten L.A., Anderson M.S., Smolenski L.A., Smith J.A., Margoliash E. & Pierce, S.K. (1987a) T cell activation by processed antigen is equally blocked by I-E and I-A restricted immunodominant peptides. *Eur J. Immunol.* **17,** 1605.

Lakey E.K., Margoliash E. & Pierce S.K. (1987b) Identification of a peptide binding protein that plays a role in antigen presentation. *Proc. natl. Acad. Sci. U.S.A.* **84,** 1659.

Lamb J.R., Feldmann M., Green N. & Lerner R.A. (1986) Influence of antigen structure on the activation and induction of unresponsiveness in clones human T lymphocytes. *Immunology*, **57,** 331.

Lin Y.L. & Askonas B.A. (1981) Biological properties of an influenza A virus specific killer T cell clone. *J. exp. Med.* **154,** 225.

Lubeck M.D., Palese P. & Schulman J.L. (1979) Non-random association of parental genes in influenza A virus recombinants. *Virology*, **95,** 269.

Maryanski J.L., Pala P., Cerrotini J. & Corradin G. (1988) Synthetic peptides as antigens and competitors in recognition by H-2 restricted gytolytic T cells specific for HLA. *J. exp. Med.* **167,** 1391.

Pala P., Bodmer H.C., Pemberton R.M., Cerottini J.-C., Maryanski J.L. & Askonas B.A. (1988) Competition between unrelated peptides recognised by H-$2K^d$ restricted cytotoxic T cells. *J. Immunol.* **141,** 2289.

Parham P., Clayberger C., Zorn S.L., Ludwig D.S., Schoolnik G.K. & Krensky A.M. (1987) Inhibition of alloreactive cytotoxic T lymphocytes by peptides from the 2 domain of HLA-A2. *Nature (Lond.)*, **325,** 635.

Taylor P.M., Davey J., Howland K., Rothbard J. & Askonas B.A. (1987) Class I MHC molecules, rather than other mouse genes dictate influenza epitope recognition by cytotoxic T cells. *Immunogenetics*, **26,** 267.

Townsend A.R.M. (1987) Recognition of influenza virus proteins by cytotoxic T lymphocytes. In: *Immunologic Research*, **6,** 80.

Townsend A.R.M. & McMichael A.J. (1987) Those images that yet fresh images beget. *Nature (Lond.)*, **329,** 42.

Townsend A.R.M., McMichael A.J., Carter N.P., Huddleston J.A. & Brownlee G.G. (1984) Cytotoxic T cell recognition of the influenza nucleoprotein and haemagglutinin expressed in transfected mouse L cells. *Cell*, **39,** 13.

Townsend A.R.M., Rothbard J., Gotch R.M., Bahadur G., Wraith D. & McMichael A.J (1986) The epitopes of influenza nucleoprotein recognised by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell*, **44,** 954.

Townsend A.R.M. & Skehel J.J. (1984) The influenza A virus nucleoprotein gene controls the induction of both subtype specific and crossreactive cytotoxic T cells. *J. exp. Med.* **160,** 552.

TOWNSEND A.R.M., TAYLOR P.M., MELLOR A.L. & ASKONAS B.A. (1983) Recognition of $D^b$ and $K^b$ gene products by influenza-sepcific cytotoxic T cells. *Immunogentics*, **17,** 283.

WATTS T.H. & MCCONNELL H.M. (1986) High affinity fluorescent peptide binding to I-Ad in lipid membranes. *Proc. natl. Acad. Sci. U.S.A.* **83,** 9660.

WINTER G. & FIELDS S. (1981) The structure of the gene encoding the nucleoprotein of human influenza virus A/PR/8/34. *Virology*, **114,** 423.

WRAITH D.C. & VESSEY A.E. (1986) Influenza virus-specific cytotoxic T-cell recognition: stimulation of nucleoprotein-specific clones with intact antigen. *Immunology*, **59,** 173.

ZWEERINK H.J., ASKONAS B.A., MILLICAN D., COURTNEIDGE S.A. & SKEHEL J.J. (1977) Cytotoxic T-cells to type A influenza virus; viral haemagglutinin induces A-strain specificity while infected cells confer cross-reactive cytotoxicity. *Eur. J. Immunol* **7,** 630.