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# Induction of single and dual cytotoxic T-lymphocyte responses to viral proteins in mice using recombinant hybrid Ty–virus-like particles

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## SUMMARY

The induction of cytotoxic T-lymphocyte (CTL) responses to viral proteins is thought to be an essential component of protective immunity against viral infections. Methods for generating such responses in a reproducible manner would be of great value in vaccine development. We demonstrate here that the recombinant antigen-presentation system based on the yeast transposon (Ty) particle-forming p1 protein is a potent means of inducing CTL responses to a variety of viral CTL epitopes, including influenza virus nucleoprotein (two epitopes), Sendai virus and vesicular stomatitis virus nucleoproteins, and the V3 loop of human immunodeficiency virus type-1 (HIV-1) gp120. CTL were primed by hybrid Ty–virus-like particles (VLP) carrying the minimal epitope or as much as 19 000 MW of protein. Ty–VLP carrying two different epitopes (dual-epitope Ty–VLP) were capable of priming CTL responses in two different strains of mice or against two epitopes in the same individual. Furthermore, co-administration of a mixture of two different Ty–VLP carrying single epitopes could induce responses to both epitopes in the same individual. Ty–VLP appear to represent a reproducible and flexible system for inducing CTL responses in mice, and warrant further evaluation in primates.

## INTRODUCTION

Until recently, the induction of cytotoxic T-lymphocyte (CTL) responses to microbial and tumour antigens has been difficult to achieve unless they are presented by some self-replicating system. This problem has hampered the development of subunit vaccines capable of stimulating strong cytotoxic responses, an important component of immunity against intracellular pathogens and malignant cells.[1–3]

Over recent years, however, a number of studies have demonstrated that protein and peptide immunogens can elicit CTL response,[4–16] and in some cases the CTL responses were protective in experimental animals if presented in an appropriate manner.[14,15] However, most of these studies employed adjuvants that are currently unlicensed for human use, although this may change for some of them in the future.[7,9] For the delivery of peptides, which can bind directly to empty major histocompatibility complex (MHC) class I molecules on antigen-presenting cells (APC), oil-based depot adjuvants have been shown to be required.[13–15] For whole proteins, requiring uptake into the MHC class I processing pathway of APC, specialized formulations designed to introduce the antigen into the cytoplasmic compartment of the cell have been used. These include immunostimulating complexes (ISCOM) and saponins.[6,7] Furthermore, the delivery of peptides into the cytosol has been achieved by linking them to hydrophobic lipid tails or fusogenic sequences.[11,17]

In addition to these specialized adjuvants and vehicles, the physico-chemical nature of the antigen is known to affect both qualitative and quantitative aspects of the immune response. Several reports have indicated that antigens presented in a particulate form can efficiently access the class I processing pathway of APC and can prime CTL responses *in vivo*. These include antigens expressed on red blood cells or liposome membranes,[8,16] latex and iron oxide beads,[18] bacteria[19] and virus-derived particles.[20]

Further evidence for the ability of particulate antigens to stimulate primary CTL responses *in vivo* came from studies on proteins which have the capacity to self-assemble into particles such as the particle-forming p1 protein encoded by the *TYA* gene of the yeast retrotransposon Ty.[21,22] When this protein is expressed in yeast, high levels of virus-like particles (Ty–VLP) can be purified from the yeast cell lysate. Protein sequences of interest can be fused to the C-terminus of p1, resulting in hybrid Ty–VLP which have been shown to be potent immunogens.[23,24] Using Ty–VLP carrying the V3 loop of the human immunodeficiency virus type-1 (HIV-1) gp120 (V3–VLP) as immunogens, we demonstrated the induction of $CD8^+$, $H\text{-}2^d$-

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restricted V3-specific CTL responses in BALB/c mice without the requirement for adjuvant. In fact, the use of alum adjuvant completely abrogated CTL induction.[25–27] Following these observations with Ty–VLP, it was shown that yeast-derived recombinant 22 nm particles formed by the hepatitis B virus surface protein (HepB S Ag) could induce HepB S Ag-specific CTL responses in BALB/c mice, and that alum adjuvant inhibited those responses.[28] Taken together these data suggest that at least some particulate protein antigens are capable of accessing the MHC class I presentation pathway of antigen-presenting cells (APC).

In this current study, we investigated the generality of the Ty–VLP system for presenting antigenic sequences containing CTL epitopes using a range of viral proteins. We have also investigated the flexibility of the system in terms of size of antigenic sequence and number/orientation of CTL epitopes.

## MATERIALS AND METHODS

*Mice*

Female BALB/c and C57BL/6 mice between 6 and 10 weeks old were obtained from Charles River Ltd (Bicester, UK).

*Peptides*

The synthetic peptides (Table 1) were purchased from Cambridge Research Biochemicals (Northwick, UK). The peptides were dissolved in sterile phosphate-buffered saline (PBS) at 1 mg/ml and stored at −80° until required.

*Hybrid Ty–VLP*

Construction and purification of hybrid Ty–VLP was as previously described.[29,30] Viral peptide sequences containing previously defined CTL epitopes (reviewed in refs 31 and 32) were selected for analysis (Table 1). *TYA* fusion genes were constructed by inserting synthetic oligomers encoding the foreign protein sequences shown in Table 1 into a yeast expression vector (pOGS40), which contains a truncated *TYA* gene encoding amino acids 1–381 of protein p1. At codon 381 there was a unique *Bam*HI site that facilitates insertion of additional coding sequences also designed to terminate in a *Bam*HI site. It was then possible to add further sequences using the terminal *Bam*HI site. As a result of this cloning strategy, all foreign sequences fused to the C-terminus of p1 were flanked by glycine-serine (GS) and glycine-serine-glycine-lysine (GSGK) amino acids. The sequences in the dual epitope constructions were spaced by GS. The resulting plasmids produced self-assembling hybrid Ty–VLP when expressed in *Saccharomyces cerevisiae* yeast. Ty–VLP were purified by sucrose density gradient followed by size exclusion chromatography. For immunization, hybrid Ty–VLP were prepared in PBS and injected intramuscularly (i.m.) (0·1 ml) into groups of mice.

In vitro *expansion of CTL effector cells*

Between 1 and 4 weeks following immunization, spleens were removed from two to three mice per group and single-cell suspensions prepared and pooled. The pooled cells were

**Table 1.** Hybrid Ty–VLP and synthetic peptides used in this study (epitopes shown in bold)

| | |
|---|---|
| HIV-1 IIIB V3: Ty–VLP (V3–VLP) | |
| p1–NCTRPNNNTRKRIRIQ**RGPGRAFVTI**GKIGNMRQAHCNIS | $ENV_{295-334}$ |
| Peptide RIQ**RGPGRAFVTI**GK ($D^d$) | |
| Influenza nucleoprotein: Ty–VLP (INP–VLP) | |
| p1–TAGLTNMMIWHSNLNDA**TYQRTRALV**RTGMDPRMCSLMQ | $INP_{130-170}$ |
| Peptide **TYQRTRALV**–TG ($K^d$) | |
| p1–**ASNENMETM** | $INP_{366-374}$ |
| Peptide **ASNENMETM** ($D^b$) | |
| Sendai nucleoprotein: Ty–VLP (SNP–VLP) | |
| p1–**APGNYPAL** | $SNP_{325-332}$ |
| p1–HGE**FAPGNYPAL** | $SNP_{321-332}$ |
| p1–HGE**FAPGNYPAL**WSYA | $SNP_{321-336}$ |
| p1–**APGNYPAL** GS **APGNYPAL** | $SNP_{325-332} \times 2$ |
| p1–RAPFICILKDPVHGE**FAPGNYPAL**WSYAMGVAVVQNKAMQ | $SNP_{309-348}$ |
| Peptide HGE**FAPGNYPAL** ($K^b$) | |
| VSV nucleoprotein: Ty–VLP (VNP–VLP) | |
| p1–**RGYVYQGL** | $VNP_{52-59}$ |
| Peptide **RGYVYQGL** ($K^b$) | |
| SNP/HIV-1 V3 dual epitope: Ty–VLP | |
| p1–**APGNYPAL** GS NNTRKRIRIQ**RGPGRAFVTI** | $SNP_{325-332}/V3_{301-322}$ |
| p1–NNTRKRIRIQ**RGPGRAFVTI** GS **APGNYPAL** | $V3_{301-322}/SNP_{325-332}$ |
| SNP/VNP dual epitope: Ty–VLP | |
| p1–**APGNYPAL** GS **RGYVYQGL** | $SNP_{325-332}/VNP_{52-59}$ |

restimulated *in vitro* in RPMI-1640 medium (10 ml) containing 10% fetal calf serum (TCM–FCS), $5 \times 10^{-5}$ M 2-mercaptoethanol (2-ME), antibiotics and 5 μg/ml peptide (Table 1). After 24 hr, a rat concanavalin A (Con A) spleen cell supernatant (RCAS) preparation[26] was added (1% final volume) as a source of growth factors. Prior to use, the RCAS was treated with saturated ammonium sulphate and the precipitate redissolved in PBS followed by extensive dialysis against PBS, then filtration through a 0·22 μm filter. This procedure effectively concentrates the growth factors and removes any residual Con A.

*Cytotoxicity assay*

After 6–7 days of *in vitro* restimulation, the effector cells (E) were washed once in TCM–FCS and 100-μl aliquots were added to 100 μl of target cells (T; $1 \times 10^4$/well) at various effector : target (E : T) ratios in triplicate or quadruplicate wells of U-bottomed 96-well microtitre plates. Target cells were P815 (H-$2^d$) or EL-4 (H-$2^b$) cells ($1 \times 10^6$/ml) incubated overnight at 37° with either 5 μg of synthetic peptide or no peptide and $^{51}$Cr (20 μCi/$10^6$ cells). Peptide-pulsed and control cells were then washed three times with TCM–FCS prior to adding to the microplate wells. After 4–5 hr, 25 μl of supernatant was removed from each well and placed in a counting plate containing 200 μl of scintillant (Hi-safe II; Wallac LKB). The plate was sealed and the contents of each well mixed thoroughly. The plates were then placed in a Microbeta Plate counter (Wallac LKB, Milton Keynes, UK). The percentage specific lysis was calculated as 100 × (test counts − mean spontaneous counts)/(mean maximum counts − mean spontaneous counts). Maximum release was generated by adding 100 μl of 5% Triton-X 100 to 100 μl target cells. Spontaneous release from the various target cells did not exceed 20%. The SEM of replicate wells did not exceed 10% for specific lysis values of > 15%. Results are expressed as net percentage specific lysis (peptide-pulsed target percentage specific lysis − control target percentage specific lysis).

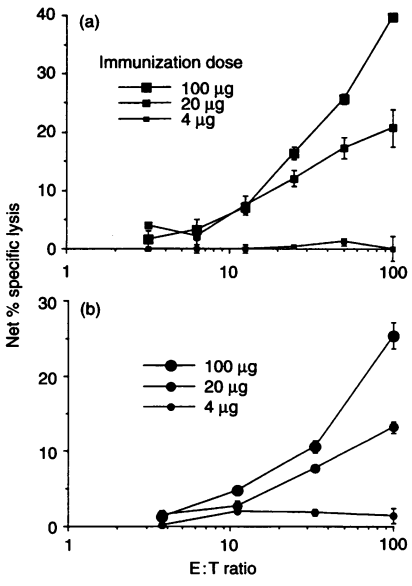


**Figure 1.** (a) $INP_{147-155}$-specific CTL responses at day 23 in BALB/c mice immunized with $INP_{130-170}$–VLP at three doses. (b) $INP_{366-374}$-specific CTL responses at day 16 in BALB/c mice immunized with $INP_{366-374}$–VLP at three doses.

## RESULTS

### Induction of CTL responses to influenza virus nucleoprotein (INP)

Influenza (strain A/PR/8/34) NP peptide (TYQRTRALV, residues 147–155) represents a CTL epitope recognized by BALB/c (H-$2^d$ haplotype) mice. Oligonucleotides encoding a peptide of 40 amino acids (residues 130–170) of INP were cloned into the *Bam*HI site of the pOGS40 yeast expression vector. The $INP_{130-170}$ sequence fused to the C-terminus of p1 is shown in Table 1. Yeast cells were transformed with this construction and the VLP purified as described in the Materials and Methods. BALB/c mice were immunized with $INP_{130-170}$–VLP at doses of 100, 20 or 4 μg without adjuvant. Spleens were removed from mice 23 days following immunization and the splenocytes restimulated *in vitro* with the $INP_{147-158.R156}$ peptide (TYQRTRALV–TG) for 7 days. This $R_{156}$ peptide has been shown to be significantly more effective in sensitizing target cells than the native $INP_{147-158}$ sequence.[33] When the effector cells were tested against P815 cells pulsed with $INP_{147-158.R156}$ peptide and control P815 cells, a dose-dependent $INP_{147-155}$-specific CTL response was seen (Fig. 1a). In order to determine whether hybrid Ty–VLP could prime CTL responses in strains of mice other than BALB/c, Ty–VLP carrying the $D^b$-restricted influenza nucleoprotein epitope ASNENMETM ($INP_{366-374}$) were produced. C57BL/6 mice were immunized with $INP_{366-374}$–VLP at three dose levels as above. Spleens were removed on day 16 and the splenocytes restimulated *in vitro* with the $INP_{366-374}$ peptide (ASNENMETM) for 7 days. When the effector cells were tested against EL-4 cells pulsed with the $INP_{366-374}$ peptide and control EL-4 cells, a dose–response was seen (Fig. 1b). Therefore Ty–VLP primed INP-specific CTL responses in two different strains of mice.

### Induction of CTL responses to the V3 loop HIV gp120

We have previously shown that hybrid VLP carrying 40 amino acids (residues 295–334) representing the V3 loop of HIV-1 gp120 (V3–VLP) can effectively induce V3-specific CTL responses in BALB/c mice.[25–27] In order to establish whether

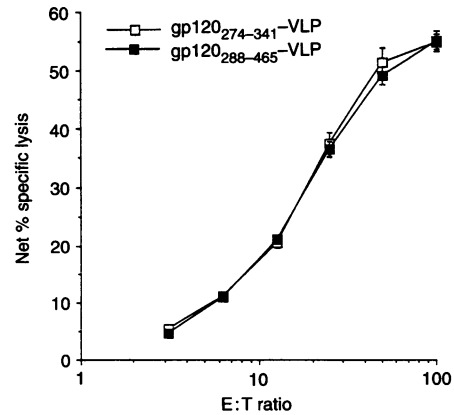


**Figure 2.** Glycoprotein 120 V3-specific CTL responses at day 9 in BALB/c mice immunized with 50 μg VLP carrying either gp120 (274–341, 67 amino acids) or gp120 (288–564, 177 amino acids).

the size of foreign sequence fused to the C-terminus of p1 affects CTL induction, we produced hybrid Ty–VLP carrying either 67 (residues 274–341) or 177 (residues 288–465) amino acids of gp120. Both of these sequences contain the $D^d$-restricted epitope RGPGRAFVTI (residues 311–320). Good yields of both hybrid Ty–VLP were produced, demonstrating that the p1 protein can tolerate at least an additional 177 amino acids (approximately 19 000 MW) of gp120 and still retain the ability to form VLP in yeast.

Mice were immunised with 50 $\mu$g of either $gp120_{274-341}$–VLP or $gp120_{288-465}$–VLP. On day 9, the spleens were removed and splenocytes restimulated with V3 peptide (RIQRGPGRAFVTIGK) for 7 days *in vitro*. The effector cells were then tested for CTL activity as previously described. Fig. 2 shows that both hybrid Ty–VLP induced strong V3-specific CTL responses.

### Induction of CTL responses to Sendai virus nucleoprotein (SNP)

In order to investigate the CTL-inducing activity of hybrid Ty–VLP in H-$2^b$ mice, and the effect of additional sequence length on CTL induction, a series of five hybrid Ty–VLP was produced, each containing the defined CTL epitope from SNP (APGNYPAL, residues 325–332) recognised by C57BL/6 (H-$2^b$) mice. The SNP sequences are shown in Table 1.

C57BL/6 mice were immunized with each SNP–VLP at a single 20 $\mu$g dose. After 21 days, splenocytes from mice were restimulated *in vitro* for 7 days with the 12-mer SNP peptide (HGEFAPGNYPAL) and then tested against SNP peptide-pulsed or control EL-4 target cells.

All five hybrid SNP–VLP induced SNP peptide-specific CTL responses (Fig. 3). The most efficient inducer of CTL at this dose and time-point was $SNP_{321-332}$–VLP (12-mer NP sequence). The addition of two CTL epitopes (8-mers spaced by GS) did not result in an enhancement of CTL levels over those observed in mice immunized with $SNP_{325-332}$–VLP. A dose–response experiment showed that a single dose of < 4·0 $\mu$g of

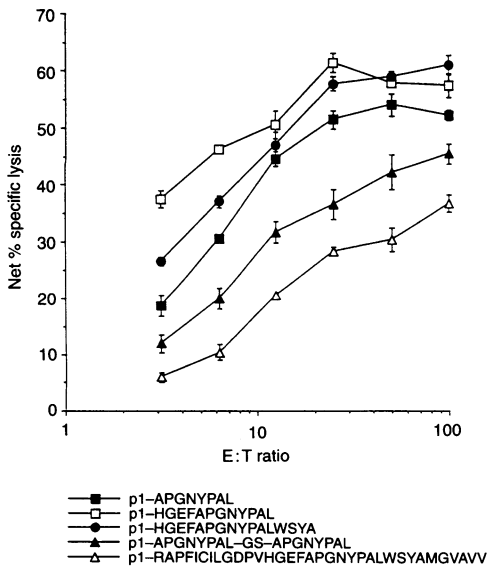


**Figure 3.** $SNP_{325-332}$-specific CTL responses at day 21 in C57BL/6 mice immunized with 20 $\mu$g VLP carrying different lengths (8–40 amino acids) of SNP.

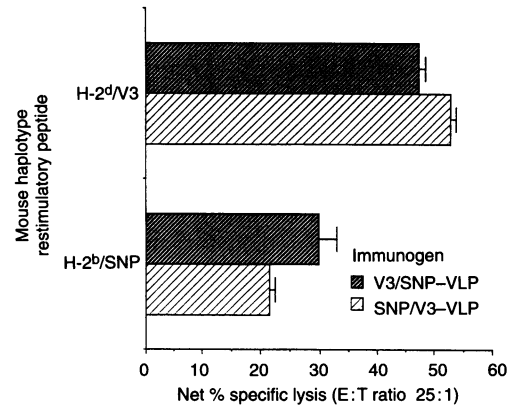


**Figure 4.** V3-specific CTL responses in BALB/c mice and SNP-specific CTL responses in C57BL/6 mice 24 days following immunization with 50 $\mu$g dual-epitope VLP carrying both the V3 and SNP epitopes (p1–V3/SNP = V3/SNP–VLP and p1–SNP/V3 = SNP/V3-VLP).

VLP carrying 40 amino acids of the SNP failed to generate CTL (net percentage specific lysis < 10% at E:T ratios of 100:1). This indicates that the peptide restimulation did not generate CTL *in vitro* in our system and that vaccination with SNP–VLP is required, as demonstrated previously using INP–VLP (Fig. 1) and V3–VLP.[25]

### Induction of CTL responses to different epitopes in two strains of mice using dual-epitope Ty–VLP

We next asked whether VLP carrying two epitopes (dual-epitope–VLP) can induce CTL in different strains of mice. VLP carrying the V3 loop (H-$2^d$) and SNP (H-$2^b$) CTL epitopes were produced. The amino acid sequences fused to the C-terminal of p1 are given in Table 1. Two Ty–VLP were produced carrying the two epitopes in different positions (p1–V3/SNP and p1–SNP/V3), each epitope separated by two amino acids (GS).

BALB/c and C57BL/6 mice were immunized with 50 $\mu$g of V3/SNP–VLP and SNP/V3–VLP. After 24 days, splenocytes were prepared from each group and restimulated *in vitro* with either V3 peptide or the SNP peptide. At day 7, the effectors were tested against the appropriate target cells pulsed with peptide, i.e. EL-4 (H-$2^b$) cells pulsed with SNP peptide and P815 (H-$2^d$) cells pulsed with V3 peptide. As shown in Fig. 4, both dual-epitope Ty–VLP were able to induce peptide-specific CTL responses in their respective mouse strains. These results demonstrate that at least two CTL epitopes, recognized by different mouse strains, can be presented on one VLP, and that the position of the epitopes may not be a critical factor for CTL induction.

### Induction of CTL responses to two epitopes in the same individuals using dual epitope Ty–VLP

Ty–VLP carrying both the $SNP_{325-332}$ (APGNYPAL) and the vesicular stomatitis virus nucleoprotein $VNP_{52-59}$ (RGYVYQGL) H-2K$^b$-restricted epitopes were produced. The two epitopes were separated by two amino acids (GS). Groups of C57BL/6 mice were immunized with 50 $\mu$g of either the $SNP_{325-332}$/$VNP_{52-59}$–VLP, $SNP_{325-332}$–VLP or $VNP_{52-59}$–VLP. After 21 days, splenocytes from each group were restimulated *in vitro*

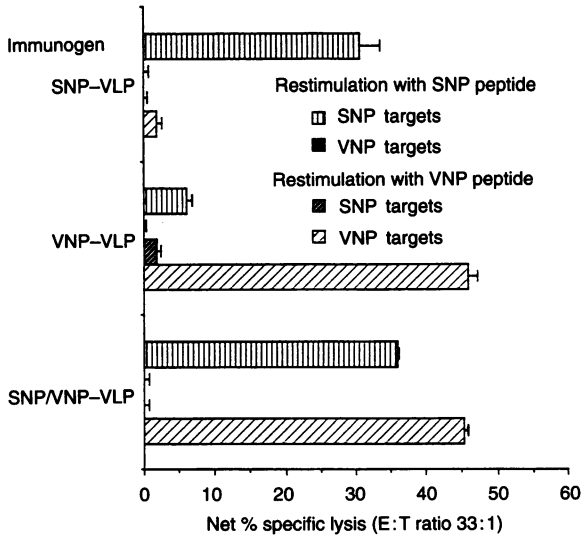


**Figure 5.** SNP- and VNP-specific CTL responses in C57BL/6 mice on day 21 following immunization with 50 $\mu$g of either dual-epitope VLP carrying both the SNP and VNP epitopes (p1–SNP/VNP = SNP/VNP–VLP) or VLP carrying the single epitopes. The error bars show SEM of replicate effectors tested against peptide-pulsed targets. The net percentage specific lysis values of SNP restimulated effectors tested against VNP targets was 0% with the three immunogens and therefore the stippled bars are not visible.

with either SNP or VNP (RGYVYQGL) peptides for 7 days prior to testing against target cells pulsed with either SNP or VNP peptides. Figure 5 shows that the dual-hybrid VLP induced CTL responses against both $SNP_{325-332}$ and $VNP_{52-59}$ and that these responses were of equivalent magnitude to those induced by the single hybrid VLP. The data also show that both *in vivo* priming and epitope-specific *in vitro* restimulation are necessary for CTL activity. The restimulation of splenocytes from mice immunized with $VNP_{52-59}$–VLP with the SNP peptide led to the generation of effector cells that showed low level killing of SNP but not VNP target cells, particularly at higher E : T ratios (100 : 1; data not shown). This may represent low-level cross-reactivity or *in vitro* priming. Splenocytes from mice primed with $SNP_{325-332}$–VLP and restimulated with the VNP peptide showed no significant killing of VNP or SNP targets at 100 : 1.

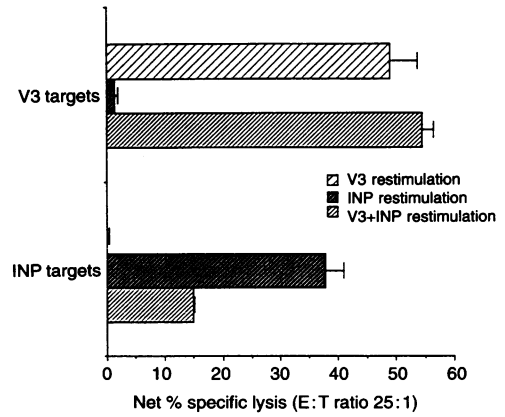


**Figure 6.** V3- and INP-specific CTL responses in BALB/c mice 14 days following co-immunization with a mixture of V3–VLP (20 $\mu$g) and INP–VLP (100 $\mu$g). The error bars show SEM of replicate effectors tested against peptide-pulsed targets.

### Induction of CTL responses to two epitopes in the same individuals by co-administration of two different hybrid Ty–VLP

Another approach to inducing CTL responses to more than one epitope is by simply mixing Ty–VLP carrying different epitopes prior to injection. BALB/c mice were immunized with a mixture of optimal doses of V3–VLP and $INP_{130-170}$–VLP (20 $\mu$g plus 100 $\mu$g) in PBS. After 14 days, splenocytes were restimulated *in vitro* with either the $INP_{147-158.R156}$ peptide (TYQRTRALV–TG) or the V3 peptide (RIQRGPGRAFV-TIGK) or both peptides for 7 days. The effector cells were then tested for CTL activity against P815 target cells pulsed with the individual peptides. Figure 6 shows that both HIV-1 V3 ($D^d$)- and INP ($K^d$)-specific CTL responses were generated in BALB/c mice using this method. Restimulation with both peptides, rather than single peptides, diminished effector cell activity against INP, but not V3 targets. This may be due to steric hindrance of binding of the INP peptide to the $K^d$ molecule by the $D^b$-binding V3 peptide, interference in the interaction between the $K^d$/INP peptide complex and the T-cell receptor, or more likely due to competition between the two expanding CTL populations for cytokines and growth factors.

### Induction of CTL responses to different epitopes in two strains of mice using chimeric Ty–VLP

In addition to dual-epitope Ty–VLP and mixing Ty–VLP, a third possibility would be to transform yeast with two plasmids expressing p1 fused to different viral sequences. This should lead to the assembly of particles containing both hybrid proteins, termed chimeric Ty–VLP. To test this hypothesis, yeast cells were co-transformed with plasmids expressing p1-$SNP_{309-348}$ and p1–$INP_{130-170}$ fusion proteins. The particles

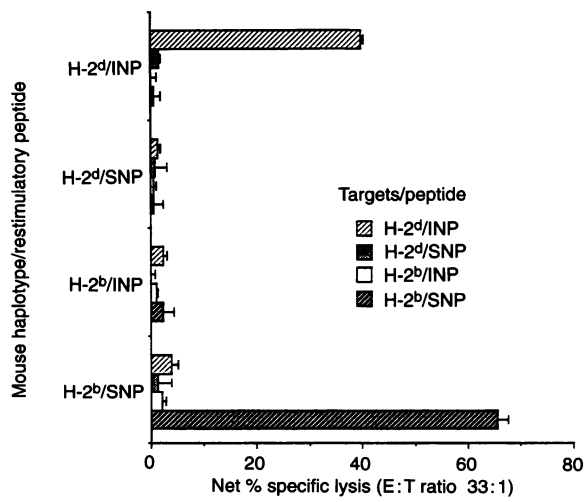


**Figure 7.** INP-specific CTL responses in BALB/c mice and SNP-specific CTL responses in C57BL/6 mice 14 days following immunization with 50 $\mu$g of chimeric VLP where particles are formed by p1–INP and p1–SNP fusion proteins. The error bars show SEM of replicate effectors tested against peptide-pulsed targets.

were purified as previously described. The relative proportions of p1–SNP and p1–INP in the VLP preparation could not be estimated because both fusion proteins give the same molecular weight band by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis. BALB/c mice and C57BL/6 mice were immunized with 50 $\mu$g of the chimeric Ty–VLP. On day 14, the splenocytes were restimulated with the SNP or INP peptides for 7 days, then tested against target cells pulsed with either SNP or INP peptides. Figure 7 shows that INP-specific CTL were induced in H-2$^d$ mice and SNP-specific CTL responses were primed in H-2$^b$ mice, as would be expected. *In vitro* priming was not observed, as in previous experiments.

## DISCUSSION

We have previously shown that hybrid Ty–VLP carrying the V3 loop of HIV gp120 can induce a strong V3-specific CTL response in BALB/c mice.[25–27] The D$^d$-restricted V3 sequence appears to be a strong epitope in that responses are induced at very low doses of V3–VLP and they persist for a long time. Many other groups have induced CTL responses to the V3 epitope in gp120 using a number of different methods[6,7,34,35] and we also observed a low V3-specific CTL response using recombinant gp120 (but not 160) without any adjuvant.[26] The data suggest that this protein sequence may possess special features facilitating efficient processing and presentation by antigen-presenting cells. There may also be a high precursor level of CTL recognizing the V3 epitope.

Given the possibility that V3 may be in some way exceptional, we tested if the Ty–VLP antigen-presentation system could reproducibly induce CTL responses to a variety of epitopes in different strains of mice. Hybrid Ty–VLP were produced, therefore, carrying well-defined CTL epitopes from various viral proteins.[31,32] Strong CTL responses were induced by these VLP to epitopes in the influenza (K$^d$ and D$^b$-restricted), Sendai (K$^b$-restricted) and vesicular stomatitis virus nucleoproteins (K$^b$-restricted), in addition to the V3 loop of HIV-1 gp120 (D$^d$-restricted). In general, high levels of CTL were generated using 10–50 $\mu$g of hybrid Ty–VLP injected intramuscularly without adjuvant. This conclusion is further supported by the recent demonstration that hybrid Ty–VLP carrying the K$^d$-restricted epitope (SYIPSAEKI) from the circumsporozoite protein ($CSP_{249-257}$) of *Plasmodium berghei*[36] also induced strong CSP-specific CTL responses in BALB/c mice (data not shown). This demonstrates that this form of antigen presentation can be employed to induce CTL responses to parasitic and, potentially, bacterial and tumour antigens.

Strong CTL responses can also be primed by subcutaneous, intraperitoneal or intranasal immunization (data not shown). The durability and magnitude of CTL responses primed by the various Ty–VLP is currently being investigated. It is interesting to note, however, that a higher dose (100 $\mu$g) of $INP_{130-170}$–VLP and $INP_{366-374}$–VLP is required to give the maximal CTL response at day 14–21. This dose is higher than for the other Ty–VLP described and the longevity of the CTL response to the $INP_{147-155}$ epitope is much shorter than that observed for the other epitopes (G.T.L., unpublished data). This may be an INP K$^d$-restricted epitope or INP protein-specific phenomenon and longevity studies of CTL primed by $INP_{366-374}$–VLP are in progress.

Ty–VLP carrying the K$^k$-restricted influenza nucleoprotein epitope (SDYEGRLI) and the Db-restricted HPV16 E7 protein epitope (RAHYNIVTF) induced only very low levels of specific CTL (data not shown) in their respective strains of mice (CBA and C57BL/6). It is unclear why these particles fail to prime strong CTL responses but there are several possibilities. Firstly, the particles failed to stimulate uptake into APC, perhaps because of the physical characteristics of the particles. Secondly, if these particles do enter the cytoplasmic compartment of APC, there may be inefficient processing of the p1 protein[37] or processing, which results in the destruction or non-generation of the MHC class I-binding epitope. This may be due to a negative influence of p1 compared to natural flanking sequences for these epitopes.[38] However, this seems unlikely because all foreign sequences fused to p1 were flanked as follows: $p1_{1-381}$GS–foreign sequence–GSGK and VLP carrying either 12 or 20 amino acids of the HPV16 E7 protein, containing the 9 amino acid epitope, were no more efficient in priming CTL than those carrying the nine amino acid epitope. Also, hybrid Ty–VLP carrying either eight, 12, 16 or 40 amino acids of the Sendai virus nucleoprotein all primed strong CTL responses.

A third possibility concerns the epitope itself, which may be a 'weak' epitope by virtue of low affinity binding to the MHC class I molecule, low affinity interaction of MHC + peptide with the T-cell receptor, or low precursor levels of CTL recognizing this epitope.

One of the perceived problems of using specific T-cell peptides in a vaccine strategy in humans is that of MHC restriction, where one peptide may only cover one haplotype. Another problem may be the requirement for responses to at least two epitopes to achieve a protective or therapeutic effect. We investigated, therefore, three strategies for presenting multiple epitopes either to different individuals or to the same individual. Firstly, the immunogenicity of hybrid Ty–VLP carrying two CTL epitopes restricted by different MHC class I molecules, the V3 (D$^d$) and SNP (K$^b$) epitopes, was evaluated. The sequences were fused to p1 as p1–V3–SNP or p1–SNP–V3. Good levels of specific CTL were detected in the appropriate strain of mouse and the levels were similar to those primed by particles carrying the single epitopes. There was an improvement in CTL responses if the epitope was presented at the C-terminus, rather than sandwiched between p1 and the other epitope. However, it is difficult to assess relative potency using a bulk culture CTL assay and only gross changes in CTL levels can be defined. These data suggest that Ty–VLP may be useful in vaccinating humans against a particular pathogen, using a 'string' of important epitopes covering prevalent MHC class I types. This 'string of beads' approach has been suggested previously.[39] Alternatively, larger sequences of protein containing multideterminant regions[40] may be fused to p1. Hybrid Ty–VLP carrying two epitopes restricted by the same MHC class I molecule (SNP and VNP; K$^b$) were also efficient in priming CTL responses to both epitopes in the same animal. This approach may be of value in vaccines designed to induce CTL responses to more than one epitope in the same protein, epitopes in different proteins from the same virus, or even epitopes in proteins from different pathogens. Somewhat suprisingly, there was no difference in the levels of response induced by particles carrying single or dual K$^b$-binding epitopes. Some degree of intramolecular

competition for MHC class I molecule binding may have been expected leading to an immunodominant response, as has been observed in class II-restricted responses,[41] particularly as the 8-mer SNP sequence may not represent the optimal epitope.[15] A dominant effect may be observed at lower VLP (epitope) concentrations and this is currently being tested. A second method for generating CTL responses to more than one epitope is to mix different VLP prior to immunization. This strategy worked well and led to the induction of $D^d$- and $K^d$-restricted CTL responses in the same individuals. A third and more sophisticated method of presenting two different epitopes is to use chimeric hybrid Ty–VLP consisting of two different p1 fusion proteins. Indeed, INP-specific responses in H-$2^d$ mice and SNP-specific responses in H-$2^b$ mice were primed using a single chimeric Ty-VLP preparation.

In conclusion, the Ty–VLP antigen-presentation system appears to be a powerful means of inducing CTL responses to microbial proteins in the absence of an adjuvant.

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