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Induction of HIV-Specific Cytotoxic T Lymphocytes In Vivo With Hybrid HIV-1 V3:Ty-Virus-Like Particles

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Abstract. In general, it has proven difficult to induce CTL responses using simple proteins or peptides without resorting to specialized adjuvants. In this study we show that particulate polymeric Ag in the form of hybrid Ty virus-like particles carrying the V3 region of HIV-1 gp120/160 envelope protein (V3:Ty-VLP) induce V3-specific CTL in BALB/c mice in the absence of adjuvant or lipid vehicle. In vitro restimulation of splenocytes with V3 peptide was necessary in order to generate effector CTL. Th cell activation was not required for this in vitro restimulation phase. The CTL induced by the V3:Ty-VLP were $CD8^+$ve, H-2^d-restricted, and HIV-1 isolate-specific (IIIB or MN). Co-administration of IIIB V3:Ty-VLP and MN V3:Ty-VLP primed both IIIB and MN V3-specific CTL. However, only IIIB V3-specific CTL were primed by hybrid Ty-VLP carrying IIIB, MN, and RF V3 loop sequences on the same particle indicating that there is intra- but not intermolecular competition between CTL epitopes. In direct comparisons, V3:Ty-VLP were substantially more potent than rgp120. Rgp160 and a 40mer IIIB V3 peptide both failed to prime V3-specific CTL. These data suggest that the particulate nature of hybrid Ty-VLP facilitates uptake into APC with subsequent access to the MHC class I processing pathway and that they may be useful vaccine vehicles for inducing cytolytic immunity against HIV-1 and other intracellular pathogens. *Journal of Immunology,* 1993, 151: 1097.

The induction of CTL has been shown to be an important component of protective immunity against viral infections in humans and mice (1–4). Although CTL responses are readily detected during viral infection or following inoculation with live recombinant vaccinia viral vectors, the ability to prime CTL in-vivo using protein or peptide immunogens has been a long sought goal. Some progress toward this goal has been made. There are several reports in which peptides have been used to induce CTL responses against Influenza, lymphocytic choriomeningitis virus, Sendai, and HIV-1 viruses in mice (5–10). However, in almost every case it has been necessary to present these peptides with adjuvants that are currently unacceptable for use in humans. In the case of whole proteins or large protein fragments there has been less success, unless the Ag is presented in a cell-associated or degraded form (11–14). There are a few exceptions. For example, recent data from Dillon et al. (15) have shown that a hybrid influenza protein, the D protein, is able to induce protective CTL responses when administered with alum adjuvant. However, in all other cases, specialized adjuvants or lipid vehicles have been essential for a significant CTL response. For example, Takahashi et al. (16) have shown that HIV-1 gp160 presented in ISCOMS[2] induces gp160-specific CTL, and similar results have been obtained by Wu et al. (17) using gp160 absorbed onto alum and administered with the

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[2] Abbreviations used in this paper: V3:Ty-VLP, V3:Ty-virus-like particles; RCAS, rat Concanavalin A-treated spleen cell supernatants; TCM-FCS, RPMI-1640 tissue culture medium containing 10% fetal calf serum; ISCOMS, immunostimulating complexes.

Table I
HIV-1 gp120 V3 loop sequences of the hybrid V3:Ty-VLP and the synthetic peptides used in this study

	V3 sequence[a]
Hybrid Ty-VLP	
IIIB V3:Ty-VLP	P1-NCTRPNNNTRKRIRIQRGPGRAFVTIGKIGNMRQAHCNIS
IIIB ΔV3:Ty-VLP	P1-NNTRKRIRIQRGPGRAFVTIGK
IIIB ΔΔV3:Ty-VLP	P1-NNTRKRIRIQRGPGR
MN V3:Ty-VLP	P1-NCTRPNYNKRKRIHI . . GPGRAFYTTKNIIGTIRQAHCNIS
IIIB/MN/RF V3:Ty-VLP	P1-IIIB V3 40mer/MN V3 39mer/RF V3 39mer
Peptides	
40mer IIIB V3	NCTRPNNNTRKRIRIQ**RGPGRAFVTI**GKIGNMRQAHCNIS
15mer IIIB V3 tip	RIQ**RGPGRAFVTI**GK
19mer IIIB V3 N-term	VEINCTRPNNNTRKSIRIQ
39mer MN V3	NCTRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAHCNIS
39mer RF V3	NCTRPNNNTRKSITKGPGRVIYATGQIIGDIRKAHCNLS

[a] Sequences taken from the Los Alamos HIV-1 database. The IIIB CTL determinant region is shown in bold.

saponin QS-21. In addition, Reddy et al. (18) have shown that specific CTL were primed in mice immunized i.v. with various Ag in liposomes, and recently it has been shown that Ag injected s.c. in a formulation of squalene and Tween 80 could prime specific CTL (19). Although the mechanisms by which these approaches lead to the induction of CTL are unknown, it has been suggested that the lipid component is the key to accessing the cytoplasmic MHC class I Ag processing pathway either by inducing cell membrane fusion with direct release of protein into the cytoplasm or by facilitating escape from the lysosomal processing pathway (16–18).

Recently, we have been evaluating the immunogenicity of particulate Ag that utilize the particle-forming p1 protein encoded by the *TYA* gene of the yeast retrotransposon Ty (20–22). In particular, we are assessing responses to Ty-VLP carrying the principal neutralizing determinant of the HIV-1 envelope protein gp120 (23, 24). This region is also known as the V3 loop. Hybrid V3:Ty-VLP are about 50 nm in diameter and are composed of approximately 300 fusion molecules. Each fusion molecule is composed of the first 381 amino acids of the Ty protein, p1, and 39 or 40 amino acids, depending on the isolate, of the HIV-1 V3 loop at the C terminus. The IIIB V3 loop sequence, therefore, comprises approximately 10% of the p1-V3 fusion protein. The fusion molecules spontaneously assemble into virus-like structures that are readily produced using a yeast expression system (25, 26). We have shown previously that hybrid V3:Ty-VLP adjuvanted with alum induce HIV-specific neutralizing antibodies and T lymphocyte proliferation (23, 24). The latter responses were seen even in the absence of adjuvant. These results prompted us to ask whether the apparent immunogenicity of these particles extended to the induction of CTL.

Materials and Methods

Mice

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

Peptides and recombinant vaccinia viruses

The MN (39mer), RF (39mer), and IIIB (40mer) V3 loop peptides were purchased from Cambridge Research Biochemicals, Inc. (Cheshire, U.K.). All other peptides (Table I) were kindly supplied by the MRC-AIDS Directed Programme. The peptides were dissolved in sterile PBS at 1 mg/ml and stored at −80°C until required. Recombinant vaccinia viruses expressing HIV-1 IIIB gp160 (vaccinia vENV2, ADP255) or HIV-1 IIIB gag (gag-vac, ADP253) were supplied by the MRC-AIDS Directed Programme.

Immunogens

Construction and purification of hybrid Ty-VLP was accomplished as previously described (25, 26). *TYA:V3* fusion genes were constructed by inserting synthetic oligomers encoding the V3 sequences shown in Table I into a yeast expression vector (pOGS40) that contains a truncated *TYA* gene encoding amino acids 1 to 381 of protein p1. The resulting plasmids produce self-assembling hybrid V3:Ty-VLP. These were purified by a sucrose density gradient followed by size exclusion chromatography. The hybrid V3:Ty-VLP contain <10 ng/mg of endotoxin as measured by the Limulus Amoebolysate test (Atlas Bioscan Ltd., Bognor Regis, UK). IIIBr gp120 produced in insect cells using the baculovirus expression system was obtained from American Bio-Technologies (Cambridge, MA). Rgp160 produced in mammalian cells using the vaccinia expression system was supplied by the MRC-ADP. The 40mer IIIB V3 peptide was used as an immunogen. The immunogens were prepared in saline and injected either i.m. (0.1 ml) or s.c. (0.2 ml) into groups of mice.

In vitro expansion of V3-specific effector cells

Between 2 and 4 wk after immunization, spleens were removed from 2 to 3 mice per group, and single cell suspensions were prepared and pooled. The pooled cells were restimulated in vitro in TCM-FCS (10 ml) containing 5×10^{-5} M 2-ME, antibiotics, and 5 μg/ml of a 40mer IIIB V3

peptide. In some experiments, a 15mer peptide representing the tip of the V3 loop and containing the H-2^d-restricted CTL epitope (16) was used at 5 μg/ml (15mer IIIB V3 tip peptide, see Table I). After 24 h, an RCAS preparation was added (2% final volume) as a source of growth factors. Before use, the RCAS was treated with saturated ammonium sulfate, and the precipitate was redissolved in PBS followed by extensive dialysis against PBS and then filtered through a 0.22 μm filter. This procedure effectively concentrates the growth factors and removes any residual Con A.

Cytotoxicity assay

After 6 to 7 days of in vitro restimulation, the effector cells were washed once in TCM-FCS and 100 μl aliquots were added to 100 μl of target cells (1×10^4/well) at various E:T ratios in triplicate wells of U-bottomed 96-well microtiter plates. Target cells were P815 (H-2^d) cells (1×10^6/ml) incubated overnight at 37°C with 20 μg/ml of the 40mer IIIB V3 peptide. This was found to be the optimal concentration for this peptide (Fig. 1*D*). In some experiments, other peptides were used (Table I) or the P815 cells were infected (4 plaque forming units/cell) with recombinant vaccinia virus expressing either gp160 (env-vac) or gag (gag-vac) for 2 h followed by one wash in TCM-FCS and an overnight incubation. Peptide-pulsed, vaccinia-infected, and control P815 cells were labeled with ^{51}Cr (1 h at 37°C, 20 μCi/10^6 cells) and then washed with TCM-FCS before being added to the microplate wells. After 4 to 5 h, 100 μl of supernatant were removed from each well for counting in a gamma counter (Beckman Gamma 5500B, Beckman Instruments, Inc., Fullerton, CA). The percentage of 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 of targets. Spontaneous release from the various targets did not exceed 20%. The SEM of triplicate wells did not exceed 10% for specific lysis values of >15%. Results are expressed as net percentage of specific lysis (peptide-pulsed target percentage of specific lysis − control target percentage of specific lysis). The control targets lysis values varied from assay to assay. In experiments using V3 peptide-restimulated effectors with high CTL activity, for example from mice immunized with V3:Ty-VLP, the control target cell values were always <5.0%, even at E:T ratios of 100:1. However, using V3 peptide-restimulated effectors with low or no specific CTL activity, for example, from normal mice or mice immunized with peptides or recombinant proteins, the control target cell values were more variable and could be as high as 20%. We attribute this nonspecific activity to NK cells, the proliferation of which appears to be suppressed when competing with Ag-driven CTL proliferation.

mAb inhibition of CTL activity

Hybridomas producing mAb to CD4 (GK1.5) or CD8 (53–6.72) were obtained from the American Type Culture Collection (Rockville, MD). Effector cells were pre-incubated with 10 μg/ml of mAb for 30 min at 37°C and then the cells were added directly to peptide-pulsed target cells. Untreated effector cells were also tested.

Lymph node proliferation assay

The assay was performed as previously described (24). Briefly, 7 days after immunization of BALB/c mice at the base of the tail, the inguinal lymph nodes were removed and a single cell suspension was prepared. The cells were then restimulated in vitro with either the 40mer IIIB V3 peptide (1 μg/ml) or rgp120 (1 μg/ml) in triplicate for 5 days. During the last 18 h of culture, tritiated thymidine (0.5 μCi/well, Amersham International, Amersham, UK) was added. Results are expressed as stimulation indices (mean dpm in stimulated cultures/mean dpm in control cultures). The error bars show SEM.

Results

V3:Ty-VLP induce potent V3-specific CTL responses in vivo

To assess the ability of IIIB V3:Ty-VLP to induce V3-specific CTL responses, groups of BALB/c mice were immunized i.m. with various doses of IIIB V3:Ty-VLP in saline, and after 18 days splenocytes were restimulated in vitro for 6 days with the 40mer IIIB V3 peptide (Table I) plus RCAS. Maximum CTL priming was observed in effector cells generated from mice immunized i.m. with a 20 μg dose (50% lysis at an E:T ratio of 10:1 and Fig. 1*A*). A 5 μg dose of IIIB V3:Ty-VLP induced a higher CTL response (50% lysis at 27:1) than did the 100- and 50 μg doses (50% lysis at 87:1 and 49:1, respectively). In vitro restimulation of normal spleen cells with the 40mer IIIB V3 peptide did not induce V3-specific CTL (data not shown and Fig. 6*A*) confirming that the CTL we are detecting are a result of in vivo rather than in vitro priming.

A series of experiments was then performed to establish the V3 specificity of the CTL response at the priming, restimulation, and target cell phases. The priming specificity of the IIIB V3:Ty-VLP was established by immunizing groups of mice with 20 μg of hybrid V3:Ty-VLP carrying either the whole 40mer IIIB V3 loop, the N-terminal 22 amino acids (ΔV3), both of which contain the defined V3 CTL determinant RGPGRAFVTI defined by Shirai et al. (27), or the N-terminal 15 amino acids (ΔΔV3), which does not contain the entire CTL determinant (Table I). After 24 days, splenocytes were restimulated in vitro with the 40mer IIIB V3 peptide for 6 days and the effector cells assayed for CTL activity. As would be expected, only mice immunized

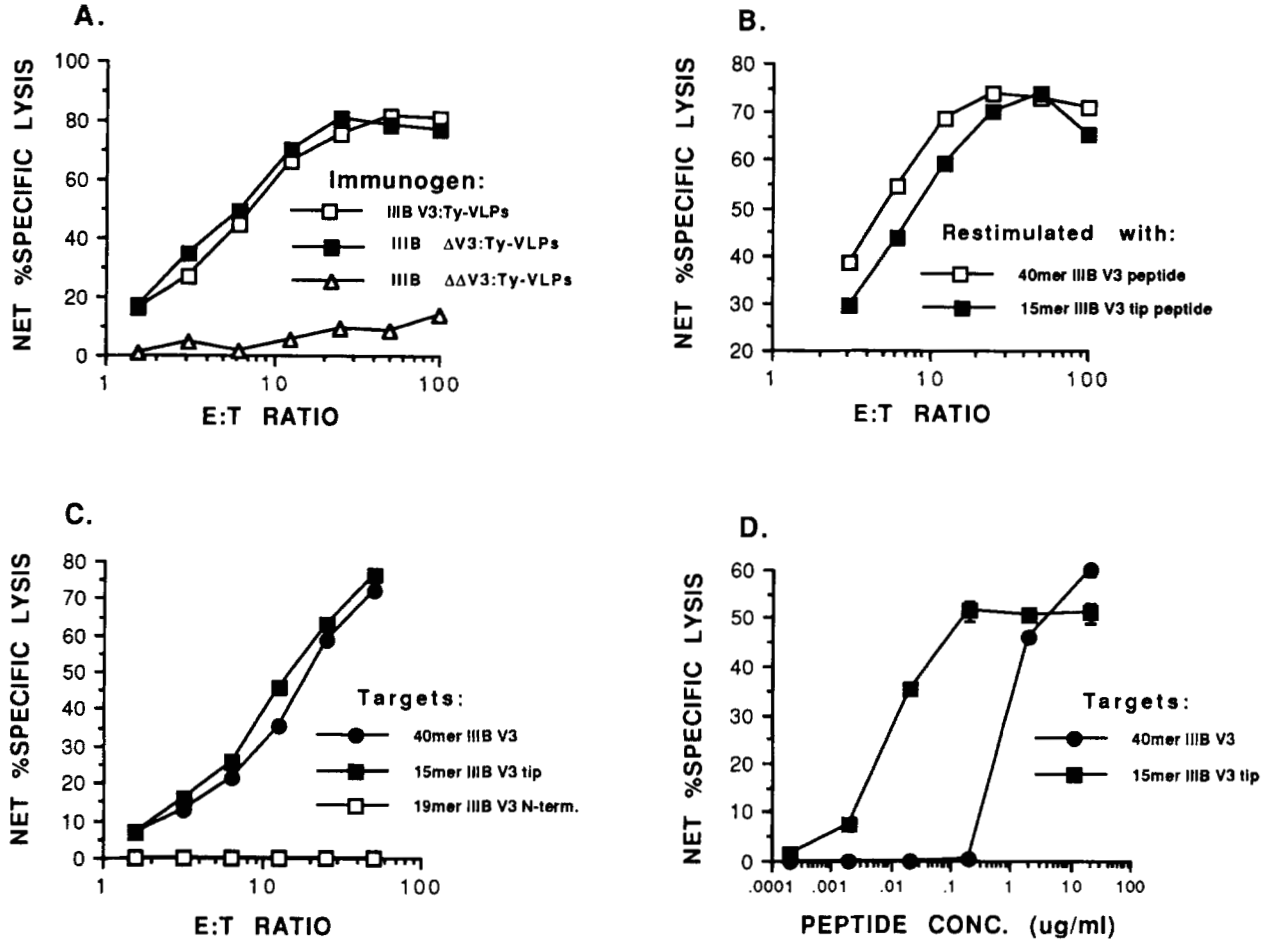

FIGURE 1. Specificity of CTL induced by IIIB V3:Ty-VLPs. *A*, Mice were immunized i.m. with 20 μg of either IIIB V3:Ty-VLP, IIIB ΔV3:Ty-VLP, or IIIB ΔΔV3:Ty-VLP. After 24 days, splenocytes were restimulated for 6 days in vitro with the 40mer IIIB V3 peptide plus RCAS and the effector cells assayed for CTL activity against ^{51}Cr-labeled P815 cells pulsed overnight with 40mer IIIB V3 peptide. *B*, Mice were immunized as above with IIIB V3:Ty-VLP and the splenocytes restimulated in vitro with either the 40mer IIIB V3 or 15mer IIIB V3 tip peptide for 6 days in the absence of RCAS. The effector cells were assayed against 40mer IIIB V3 peptide-pulsed targets as above. *C*, Effector cells generated by 40mer IIIB V3 peptide restimulation of splenocytes from IIIB V3:Ty-VLP-immunized mice were assayed for CTL activity against ^{51}Cr-labeled P815 cells pulsed overnight with 20 μg/ml of either the 40mer IIIB V3 peptide, 15mer IIIB V3 tip peptide, 19mer IIIB V3 N-terminal peptide, or no peptide. *D*, Effector cells described in *C* above were added to ^{51}Cr-labeled P815 cells and either the 40mer IIIB V3 or the 15mer IIIB V3 tip peptide was added directly to the assay at various concentrations. The E:T ratio was 50:1.

with IIIB V3:Ty-VLP or the ΔV3:Ty-VLP were primed for a V3-specific CTL response (Fig. 1*A*). In order to assess Th cell proliferative responses, BALB/c mice were immunized with the three immunogens described above, and the draining lymph node cells were restimulated in vitro with the 40mer IIIB V3 peptide. As shown in Table II, lymphocytes from mice primed with IIIB V3:Ty-VLP, but not with the two truncated V3 immunogens, proliferated in response to the 40mer IIIB V3 peptide. The responding cells have previously been shown to be CD4$^+$ (24). These data confirm that the Th epitope is in the C-terminal half of the V3 loop, as previously described (28), and suggest that Th cell activation is not required for precursor CTL activation in vitro, at least in the presence of RCAS. To confirm this observation, splenocytes from mice immunized with IIIB V3:Ty-VLP were restimulated for 6 days with either the 40mer IIIB V3 peptide containing both the CTL and Th

Table II
In vitro lymph node proliferative responses to V3 peptide and gp120 in mice immunized with hybrid V3:Ty-VLP

Immunogen	Stimulated in vitro with	
	40mer IIIB V3 peptide	gp120
IIIB V3:Ty-VLP[a]	18.2 (1.7)[b]	5.2 (0.3)
IIIB ΔV3:Ty-VLP	2.4 (0.4)	0.7 (0.06)
IIIB ΔΔV3:Ty-VLP	1.5 (0.4)	1.3 (0.3)

[a] Mice immunized with 50 μg of IIIB V3:Ty-VLP in alum adjuvant.
[b] Stimulation index (SEM).

determinants or the 15mer V3 tip peptide containing just the CTL epitope in the absence of RCAS. There was only a slight decrease in V3-specific CTL activity following restimulation with the 15mer IIIB V3 tip peptide (50% lysis at an E:T ratio of 5:1) compared to the 40mer IIIB V3 peptide (50% lysis at an E:T ratio of 8:1) indicating that Th cell activation is not required for the in vitro generation of effector CTL (Fig. 1*B*). No CTL activity was detected in fresh non-restimulated splenocytes from IIIB V3:Ty-VLP-immunized mice (data not shown) emphasizing the requirement for Ag-driven expansion of precursor CTL.

To assess the target specificity of the CTL, spleen cells from mice immunized with IIIB V3:Ty-VLP were restimulated in vitro with the 40mer IIIB V3 loop peptide and the effector cells generated were tested against P815 cells preincubated with the 40mer IIIB V3 loop peptide, a 15mer IIIB V3 tip peptide, a 19mer IIIB V3 N-terminal peptide (Table I), or no peptide. P815 cells were recognized as target cells only when pulsed with either the 40mer IIIB V3 loop peptide or the 15mer IIIB V3 tip peptide (Fig. 1*C*), both of which contain the H-2d-restricted CTL epitope. When the E:T ratio was held constant and the 15mer and 40mer IIIB V3 peptides were titrated directly in the CTL assay using IIIB V3:Ty-VLP-primed effector cells there was a 2-log difference in the concentration required to give 50% lysis (Fig. 1*D*). This may reflect differing requirements of the two peptides for either intracellular processing or extracellular degradation before being presented in the peptide groove of MHC class I molecules. The optimal length of naturally processed K^d-binding peptides was found to be nine amino acids (29), and a recent in vitro study has demonstrated that extracellular proteases can trim longer V3 peptides to more active shorter ones (30).

In order to confirm that the CTL primed by the IIIB V3-VLP can kill target cells endogenously synthesizing HIV-1 gp160, 40mer IIIB V3 peptide-restimulated effector cells were tested against P815 cells infected with either env-vac or gag-vac vaccinia viruses or against control P815 cells at an E:T ratio of 10:1. The percentages of specific lysis values of the three target cell preparations were 35.8% (env-vac), 3.1% (gag-vac), and 1.2% (non-pulsed control). These results demonstrate that the CTL primed by V3:Ty-VLP can recognize and kill cells infected with virus-expressing gp160.

CTL primed by V3:Ty-VLP are HIV-1 isolate-specific

In previous studies, CTL lines generated from mice immunized with vaccinia virus expressing gp160 from either the IIIB, MN, or RF HIV-1 isolate have been shown to be isolate-specific. For example, IIIB-specific CTL do not kill MN targets and vice versa (31–33). In our studies, effector cells primed by IIIB V3:Ty-VLP were isolate-specific in that they did not recognize P815 cells pulsed with peptides

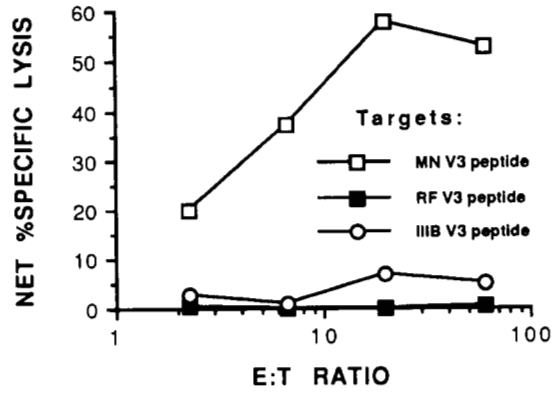

FIGURE 2. MN V3:Ty-VLP prime MN V3-specific CTL. BALB/c mice were immunized i.m. with 100 μg of MN V3:Ty-VLP. On day 18, splenocytes were restimulated in vitro for 6 days with 39mer MN V3 loop peptide (5 μg/ml). E were assayed against ^{51}Cr-labeled P815 cells pulsed overnight with 20 μg/ml of either the 40mer IIIB, 39mer MN, or 39mer RF V3 loop peptide, or no peptide.

representing the V3 loop sequences of MN or RF isolates (specific lysis <5% at 100:1 E:T ratio). To ascertain if hybrid V3:Ty-VLP expressing the V3 region from other isolates of HIV-1 could prime V3-specific CTL, BALB/c mice were immunized i.m. with MN V3:Ty-VLP in saline, and splenocytes were restimulated in vitro with a 39mer MN V3 peptide (Table I). Substantial levels of CTL activity were detected against P815 cells pulsed with the 39mer MN but not with the 40mer IIIB or 39mer RF V3 peptides (Fig. 2).

The V3 loop region of gp160 from different isolates of HIV-1 is highly variable and, as described above, this leads to the generation of isolate-specific CTL. Because of the need to accommodate some of this variability in any potential vaccine, we investigated the effects of co-administering two V3 sequences (MN and IIIB) carried on different particles and the effect of presenting three different V3 sequences (IIIB, MN, and RF) on the same particle. Immunization of BALB/c mice with a mixture of MN and IIIB V3:Ty-VLP led to the induction of both MN- and IIIB-specific CTL responses (Fig. 3). There was no evidence of cross-reactivity in that the peptides only expanded CTL recognizing the homologous isolate. Interestingly, when mice were immunized with hybrid Ty-VLP carrying the IIIB, MN, and RF V3 loop sequences on the same particle (p1-IIIBV3/MNV3/RFV3 fusion protein forming IIIB/MN/RF V3:Ty-VLP) and with the splenocytes restimulated in vitro with the 40mer IIIB, 39mer MN, or 39mer RF V3 peptides, only IIIB V3-specific CTL were detected (Fig. 4).

V3:Ty-VLP prime $CD8^+$ve, H-2d-restricted CTL

Previous studies using both exogenous (V3 peptides or gp160 in various adjuvants) and endogenous (vaccinia

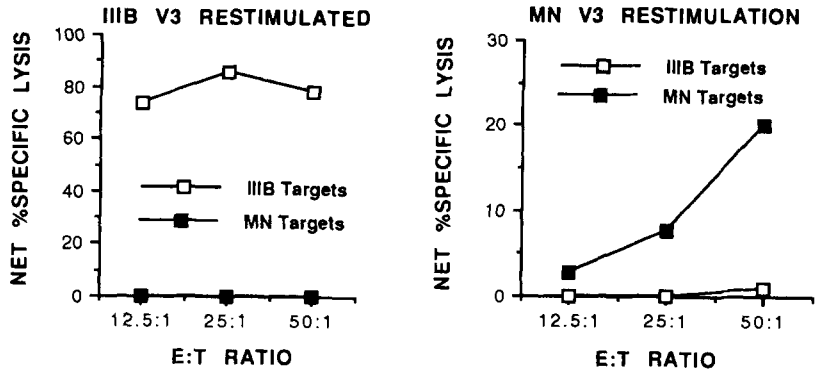

FIGURE 3. Immunization with IIIB and MN V3:Ty-VLP primes both IIIB and MN V3-specific CTL. Mice were immunized i.m. with a mixture of IIIB and MN V3:Ty-VLP (20 μg each). After 21 days splenocytes were restimulated in vitro with either the 40mer IIIB or 39mer MN V3 peptide (5 μg/ml) for 6 days. Effectors were assayed against ^{51}Cr-labeled P815 cells pulsed overnight with either the 40mer IIIB or 39mer MN V3 peptide.

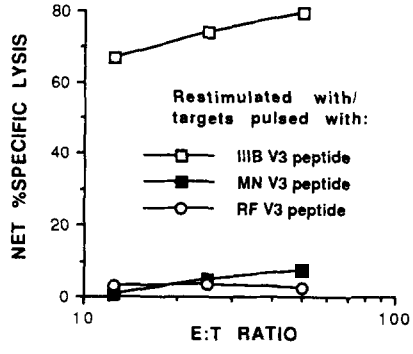

FIGURE 4. IIIB/MN/RF V3:Ty-VLP prime only IIIB V3-specific CTL. Mice were immunized i.m. with IIIB/MN/RF V3:Ty-VLP (100 μg) and after 24 days splenocytes were restimulated in vitro with the 40mer IIIB, 39mer MN, or 39mer RF V3 peptide for 7 days. Effector cells were assayed against ^{51}Cr-labeled P815 cells pulsed overnight with either the IIIB, MN, or RF V3 peptides.

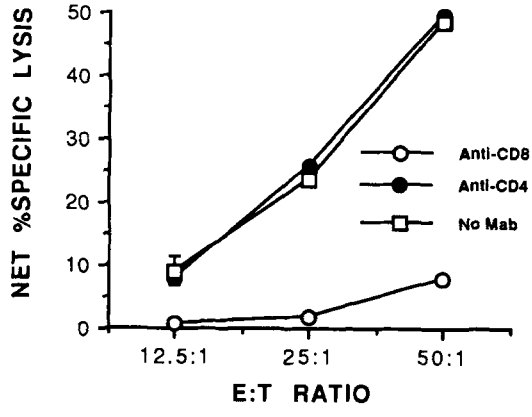

FIGURE 5. V3:Ty-VLP induce CD8$^+$ V3-specific CTL. MN-specific effector cells generated as described in Figure 2 were preincubated with anti-CD4, anti-CD8 (10 μg/ml), or no mAb for 30 min, and the cells then were added to ^{51}Cr-labeled P815 cells pulsed overnight with 39mer MN V3 peptide.

virus-gp160) immunogens have shown that V3-specific CTL responses in mice are CD8$^+$ and H-2D^d-restricted (10, 16, 19, 31, 32). We wished to confirm that this is also true for CTL primed by V3:Ty-VLP. When effectors from MN V3:Ty-VLP-primed mice were incubated with anti-CD4 and anti-CD8 mAb and then added directly to the CTL assay, inhibition of V3-specific CTL activity was observed only in the cultures treated with anti-CD8 antibody demonstrating that the effector cells are CD8$^+$ (Fig. 5). Furthermore, when C57BL/6 (H-2^b) and BALB/c (H-2^d) strains of mice were immunized with V3:Ty-VLP, CTL activity was not detected using EL-4 (H-2^b) cells as targets, indicating that the V3-specific CTL are restricted at the H-2^d locus as previously described.

Comparative immunogenicity of V3:Ty-VLP and non-particulate V3 loop Ag

To ask if the particulate nature of the Ag is important in the induction of CTL, we compared the ability of IIIB V3:Ty-VLP, 40mer IIIB V3 peptide, and rgp120 (rgp120) to induce CTL. BALB/c mice were immunized i.m. with the three types of V3 immunogen. The IIIB V3:Ty-VLP were also injected s.c. Effector cells from mice immunized i.m. with IIIB V3:Ty-VLP gave 50% lysis at an E:T ratio of 4:1, whereas the s.c. route was less efficient with 50% lysis at 10:1 (Fig. 6A). The rgp120 was approximately 25-fold less potent than the IIIB V3:Ty-VLP in priming CTL, and effectors from mice immunized with the 40mer IIIB V3 peptide only produced 10.3% killing at an E:T ratio of 100:1 at the 100 μg dose. In a separate experiment, in vitro restimulated splenocytes from mice immunized with a mixture of non-hybrid Ty-VLP (20 μg) plus the 40mer IIIB V3 peptide (2 μg or 100 μg) failed to show significant CTL activity at an E:T ratio of 100:1. This demonstrates that Ty-VLP do not simply deliver nonspecific signals enabling CTL induction and that immunogenicity of the V3-VLP is completely dependant upon the V3 loop being presented as a p1-V3 fusion protein in a particulate form. The response induced by the rgp120, albeit a low level response, was somewhat surprising inasmuch as rgp160 had previously been shown not to induce CTL responses in the absence of ISCOMS (16), although in that study, doses of only 1 μg of Ag were used. Further dose response studies on IIIB

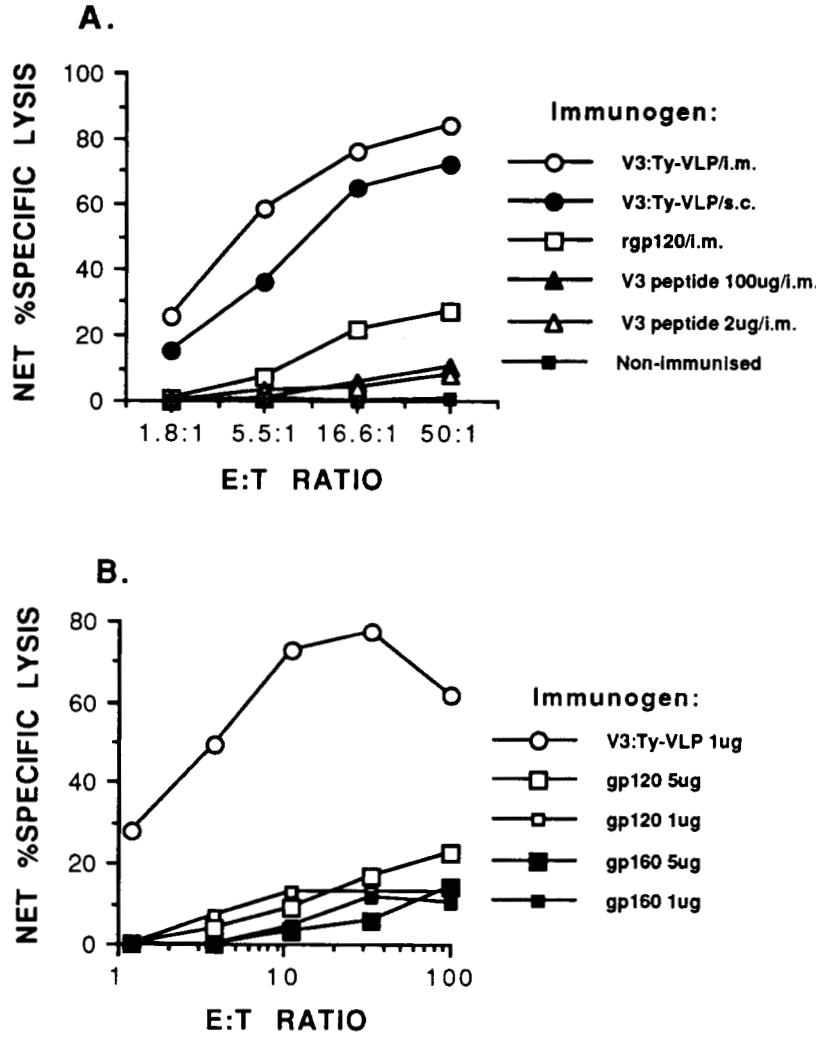

FIGURE 6. Hybrid IIIB V3:Ty-VLP are superior to 40mer IIIB V3 peptide, rgp120 and rgp160 in priming V3-specific CTL responses. *A*, BALB/c mice were immunized i.m. with 40mer IIIB V3 peptide at 100 μg or 2 μg, rgp120 at 20 μg, IIIB V3:Ty-VLP at 20 μg, or IIIB V3:Ty-VLP at 20 μg injected s.c. Non-immunized mice served as controls. On day 25, pooled splenocytes were restimulated for 6 days with 40mer IIIB V3 peptide and effector cells assayed against 40mer IIIB V3 peptide-pulsed or control P815 cells. *B*, Mice were immunized i.m. with IIIB V3:Ty-VLP (1 μg), rgp120 (1 and 5 μg), or rgp160 (1 and 5 μg). After 24 days, splenocytes were restimulated for 6 days with 40mer IIIB V3 peptide, and effector cells were assayed against ^{51}Cr-labeled P815 cells pulsed overnight with the 40mer IIIB V3 peptide.

V3:Ty-VLP, rgp120, and rgp160 revealed that as little as 1.0 μg of IIIB V3:Ty-VLP induced a strong CTL response following in vitro restimulation (Fig. 6B). Fifty per cent lysis was observed at an E:T ratio of 3.7:1. In contrast, rgp160 did not induce a significant CTL response using a 1 or 5 μg dose confirming the previous data (16). Rgp 120, however, induced a detectable response at a dose of 5 μg, but not 1 μg, only at the highest E:T ratio.

Discussion

Several points emerge from this study, the most important being that hybrid V3:Ty-VLP are able to induce a potent HIV-specific CTL response in mice. This could have major implications for HIV vaccine design. Preliminary experiments indicated that too high a dose of V3:Ty-VLP may have a deleterious effect on CTL priming. This may be due either to a direct effect on APC at the site of injection or an effect on other cells required to facilitate CTL priming. It is not clear whether the lower responses observed at higher doses represent a permanent suppression or simply a change in the kinetics of the response. Studies are now in progress to investigate this issue. $CD4^+$ T cell help is known to be required for in vivo priming of CTL (34, 35) and maintenance of CTL activity (9). Both V3 and p1 (Ty)--specific $CD4^+$ Th cells are primed by IIIB V3:Ty-VLP (24). It has been suggested that the 15mer IIIB V3 peptide containing the CTL determinant can induce $CD4^+$ T cell help for itself (36), and this peptide efficiently restimulated V3-specific CTL in vitro in our studies. However, when this sequence was presented to BALB/c mice in the form of IIIB ΔV3:Ty-VLP, a V3-specific proliferative T cell response was not detected. This suggests that this sequence does not contain an H-2^d class II-restricted epitope, and that T cell help is not required for the in vitro generation of effector CTL from a population of primed CTL precursors as previously described (35).

Whereas it has been easy to detect HIV-specific CTL in splenocytes restimulated in vitro, we have so far failed to detect CTL in fresh, non-restimulated splenocytes. This suggests that immunization with V3:Ty-VLP leads to the

proliferation of precursor CTL that require additional stimuli, and possibly differentiation, before they are capable of killing appropriate target cells. Boosting with V3:Ty-VLP in vivo does not appear to provide this stimulus (data not shown). This apparent lack of killing by fresh splenocytes may reflect an operational mechanism for preventing primed CTL from killing APC. This would make sense in terms of maintenance of immune responses by APC capable of initiating both class I- and class II-restricted responses, for example, in dendritic cells (37, 38). Recent data have shown that CTL can inhibit class I- and class II-restricted Ag presentation in vitro by specialized APC from the spleen of mice (39). It is also possible that activated, but not resting, CTL can kill APC in vivo, and we are unable to detect the former in fresh cultures because of the very low precursor frequency. It is also conceivable that CTL are switched on for lysis in vivo only when encountering virally infected or transformed cells expressing class I-restricted epitopes. It is interesting to note that high levels of CTL activity were also detected in PBL of IIIB V3:Ty-VLP-immunized mice following restimulation in vitro (data not shown).

Using as little as 1 μg, IIIB V3:Ty-VLP stimulated a potent CTL response against a determinant at the tip of the V3 loop. This is the same region that is recognized by CTL induced by both gp160 in ISCOMS (16) and vaccinia virus expressing gp160 (31). Within this region the CTL epitope has recently been resolved down to the decapeptide RGPGRAFVTI (27). The CTL induced are $CD8^+$, $H\text{-}2^d$-restricted and can kill infected with vaccinia virus expressing gp160 (31). Recent studies have shown that class I molecules from three other mouse haplotypes ($H\text{-}2^p$, $H\text{-}2^u$, and $H\text{-}2^q$) can also present the 15mer IIIB V3 tip peptide for CTL recognition (27) and, significantly, this V3 loop peptide was also found to be recognized by CTL from 8/20 HIV-1 seropositive patients of different MHC haplotypes when presented on EBV-transformed autologous target cells (40). This permissiveness in MHC class I binding suggests that this epitope may be an important candidate for inclusion in an HIV-1 vaccine.

There is now a growing body of evidence in the MHC class II Ag presentation system to indicate that there are multiple factors involved in the mechanism of epitope immunodominance (41). Such factors include intramolecular competition between T cell epitopes for the same MHC class II molecule (42) and intermolecular competition between different MHC class II molecules (I-A- and I-E-) for T cell epitopes on the same molecule (43). Both of these types of dominance are dependent on the context of the epitope within the molecule and are not simply a function of processing or MHC-binding affinity. Evidence would suggest that there may also be competition between class I and class II molecules for peptide binding inasmuch as identical peptides can be recognized by both (44). We were interested, therefore, in assessing immunodominance in the class I Ag presentation system using V3:Ty-VLP carrying multiple V3 loop sequences known to be recognized in a $H\text{-}2D^d$-restricted manner. Our data reveal that intramolecular immunodominance exists in class I-restricted responses and that the concept of producing synthetic vaccines displaying multiple CTL epitopes that utilize the same MHC restriction element may not be feasible. When two epitopes were presented to mice on different Ty-VLP, CTL responses were detected against both epitopes. This suggests that a cocktail approach to vaccination may be possible.

The mechanism behind the intramolecular immunodominance is unknown. In addition to intracellular processing and presentation events, extracellular processing may be a contributory factor in determining which epitope is eventually presented to the T cell. Recent in vitro studies have shown that extracellular processing of the 15mer IIIB V3 tip peptide by angiotensin-1-converting enzyme results in the generation of a more active peptide in terms of MHC-binding and CTL activation (30). One could speculate that similar cleavage events may trim sequences from the Ty-VLP hybrid p1-IIIBV3/MNV3/RFV3 protein resulting in loss of the MN and RF V3 sequences before uptake into the APC. It is interesting to note, however, that the 40mer IIIB V3 loop peptide, either alone or mixed with non-hybrid Ty-VLP, was ineffective in priming CTL in vivo, although the CTL epitope within this peptide is efficiently presented by P815 cells for CTL recognition in vitro. This indicates that the conditions prevailing in vitro may not operate in vivo, suggesting that if class I-binding peptides are generated by extracellular peptide or protein processing, then other costimulatory signals are required for CTL priming and the presence of the non-hybrid Ty-VLP did not provide those signals. We have shown that the injection of the V3 peptide in Freund's complete adjuvant can lead to a V3-specific CTL response (unpublished observation) demonstrating that the peptide is capable of priming CTL under the right conditions.

Our results demonstrate that particulate V3:Ty-VLP are substantially more potent than non-particulate V3 loop immunogens, although we cannot rule out the possibility that this may be due to differences in polypeptide sequence or length rather than physical state. Our data suggest that hybrid V3:Ty-VLP can gain access to the class I processing pathway of APC in vivo much more efficiently than non-particulate proteins and peptides. In fact, there has been only one report describing the induction of class I-restricted CTL responses using soluble monomeric protein, and this study could not rule out the possibility that aggregated protein or degraded protein fragments were the active components (12).

The CTL priming effect of V3:Ty-VLP may be due to one or more of several factors. First, it may be due to optimal stimulation of endocytosis or phagocytosis leading to efficient uptake into specialized APC in the spleen (39) or at

the site of injection. It has been suggested recently that dendritic cells are the main cell type responsible for CTL priming (37–39), and that macrophages may be involved in pre-processing certain Ag before passing on material to dendritic cells (37, 45). Second, following uptake into the APC, subsequent leakage of class I-binding V3 peptides from the lysosomal compartment into the cytoplasm may occur. There is a precedence for dual lysosomal and cytoplasmic processing for class II and class I presentation using acid-sensitive liposome-encapsulated Ag (46). However, liposomes are thought to gain access to the cytoplasm via liposome-endosome fusion, and because Ty-VLP do not contain any lipid, this does not seem to represent a likely mechanism.

Third, V3:Ty-VLP may access the cytoplasm directly via membrane penetration. This is thought to be a possible mechanism of entry of Ag presented either in ISCOM, with saponins, or as lipopeptides (5, 36). However, as already stated, V3:Ty-VLP do not contain lipid nor do they possess any obvious membrane attack or fusion activity like that of the saponins or viral envelope fusion proteins. There are several reports indicating that the V3 loop of HIV-1 gp160 is a domain which facilitates cell fusion (47). However, even conservative mutations within the V3 loop in gp160 abrogate its ability to induce cell fusion (48), and the ΔV3: Ty-VLP, which carry only the central 22 residues of the loop, are as potent immunogens as Ty-VLP carrying the whole V3 loop. Also, gp120 was poorly immunogenic.

The fourth alternative is that the particles may be degraded and simply provide peptide fragments that can bind to empty class I molecules on the surface of the cell. This possibility seems unlikely. Degradation might occur either before or after immunization. However, if degradation occurs before immunization, then it must be at an undetectably low level (data not shown) and could not account for the in vivo results, because as we know that even 100 μg of V3 peptide without adjuvant fails to prime a significant response. If extracellular degradation occurs post-immunization, then the difference in efficacy between V3: Ty-VLP, the recombinant proteins, and V3 peptide would need to be explained by selective degradation of the Ty-VLP. This is formally possible but unlikely. Studies are now in progress to attempt to define the mechanism of CTL induction by particulate proteinaceous particles.

Whatever the mechanism of induction of HIV-specific CTL by V3:Ty-VLP, it is clear that if this system can elicit such a response in humans, then it may be of value for vaccination against HIV-1 infection. Furthermore, hybrid Ty-VLP may represent a useful vaccine vehicle for immunotherapeutic intervention during other viral infections and possibly in malignancy.

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