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# Enhanced proliferative cellular responses to HIV-1 V3 peptide and gp120 following immunization with V3:Ty virus-like particles

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## SUMMARY

The induction of CD4+ T-helper (Th) cell responses is likely to be an important requirement of vaccine candidates designed to prevent or moderate human immunodeficiency virus-1 (HIV-1) infection. We have investigated the ability of hybrid Ty virus-like particles carrying the V3 loop region of the HIV-1 IIIB envelope gp120 (V3:Ty-VLP) to elicit V3-specific proliferative responses. Significant proliferation in response to stimulation *in vitro* with homologous IIIB V3 peptide was observed following immunization of mice with V3:Ty-VLP either as an aluminium hydroxide precipitate or without adjuvant. Responses to MN V3 peptide were also observed in certain mouse haplotypes. To assess the effect of presenting the V3 loop in this particulate form, we compared the responses induced by V3:Ty-VLP with those obtained with two non-particulate immunogens, recombinant gp120 (rgp120) and V3 peptide conjugated to albumin. V3-specific responses to V3 peptide *in vitro* were reproducibly higher following immunization with V3:Ty-VLP than with either rgp120 or V3-albumin coagulate (V3-alb). The data indicate that immunization with the V3 loop as a hybrid Ty-VLP results in enhanced proliferative responses to V3 peptide and recognition of rgp120 *in vitro*. Some cross-reactivity of Th cells for V3 sequences from different isolates was also observed.

## INTRODUCTION

A successful vaccine must induce immunological memory such that protective responses are achieved upon exposure to the invading organism. This requires the generation of antigen-specific memory T and B lymphocytes. In the development of a vaccine against human immunodeficiency virus-1 (HIV-1) it is therefore important to consider both humoral and cellular components of the immune response. Several laboratories have demonstrated the induction of neutralizing antibodies against HIV envelope glycoproteins[1–5] and there is increasing evidence that neutralizing antibodies are likely to play a key role in a protective immune response.[6] In addition, the generation of a potent cytotoxic T-lymphocyte (CTL) response by either prophylactic or therapeutic vaccine candidates may be important, as indicated by the increased long-term survival of HIV+ individuals who have a strong CD8+ T-lymphocyte activity against HIV-infected cells.[7] The activation of CD4+ T lymphocytes results in the production of cytokines which enhance CTL function, promote differentiation of B lymphocytes into plasma cells and increase the frequency of memory B lymphocytes.[8] The induction of HIV-specific CD4+ T-helper (Th) lymphocyte responses will therefore be an essential requirement for any HIV vaccine.

A number of Th-cell epitopes have been identified in the envelope glycoproteins of HIV.[9–11] At least two of these epitopes are contained within the disulphide-cross-linked third variable domain (V3 loop) of gp120.[10,11] The V3 loop also contains epitopes recognized by CTL in both mice and humans,[12,13] and the principal antibody neutralization determinant (PND) of HIV-1.[1] We have shown previously that fusion proteins comprising of the p1 protein of the yeast transposon Ty and a V3 loop sequence from the HIV-1 isolate IIIB assemble into 50 nm virus-like particles (V3:Ty-VLP) containing approximately 300 copies of the p1:V3 fusion protein.[14] The V3:Ty-VLP have been shown to induce high titre anti-HIV neutralizing antibodies in rabbits and HIV-specific T-cell proliferative responses in mice.

To evaluate the efficiency of presenting the V3 region in this particulate form, we have compared the lymph node proliferative responses obtained following immunization with V3:Ty-VLP with those seen using V3 in non-particulate form, either contained within recombinant gp120 (rgp120) or as a V3-albumin conjugate (V3-alb). We have also studied the effects of alum adjuvant on the magnitude of proliferative responses towards the V3 loop. Finally, we have investigated the ability of rgp120 and also the V3 loop peptide from the MN HIV-1 isolate

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to induce proliferation in lymphocytes from V3:Ty-VLP-primed mice.

## MATERIALS AND METHODS

*Mice*

C57BL/6 (H-$2^b$), BALB/c (H-$2^d$), C3H/Hej (H-$2^k$) and DBA/2 (H-$2^d$) female mice were obtained from Charles River (Margate, U.K.). CBA (H-$2^k$) and A.SW (H-$2^s$) female mice were obtained from Harlen Olac (Bicester, U.K.). Mice were approximately 7–10 weeks old.

*Immunization schedule*

Immunogens were injected subcutaneously near the base of the tail in a volume of 0·1 ml as an aluminium hydroxide (alum) precipitate, unless otherwise stated.

*Immunogens*

A 40-mer peptide corresponding to the V3 loop of HIV-1 isolate IIIB, clone HXB2 (IIIB V3 peptide),[15] was obtained from Cambridge Research Biochemicals (Cheshire, U.K.) with the following sequence:

NCTRPNNNTRKRIRIQRGPGRAFVTIGKIGNMRQAHCNIS

For some experiments the peptide was conjugated to bovine serum albumin (BSA) using gluteraldehyde as previously described[16] (V3-alb). The presence of immunoreactive V3 sequences in the V3-alb conjugate was confirmed by immunoblotting with an anti-V3 antibody. Also, V3-alb was able to stimulate proliferative responses in cells from mice immunized with V3:Ty-VLP to the same degree as V3 peptide alone (data not shown).

A V3 peptide corresponding to the MN (MN V3 peptide) sequence was obtained from Cambridge Research Biochemicals, with the following sequence:

NCTRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAHCNIS

Insect-derived recombinant gp120 (rgp120) from isolate IIIB, clone BH10 was obtained from American Bio-Technologies (Cambridge, MA). The V3 region within the BH10 gp120 sequence differs by one amino acid residue from that of clone HXB2 (R⇒S), as shown underlined on the IIIB peptide sequence above. The V3 loop sequence comprises 9·1% of the molecular weight of the protein of rgp120.

Ty-VLP containing the V3 loop region from the clone HXB2 (V3:Ty-VLP) were constructed and purified as previously described.[17] The V3 loop sequence comprises 10·5% of the molecular weight of the Ty:V3 fusion protein.

*Monoclonal antibodies*

Monoclonal antibodies were derived from cell culture supernatants of the hybridomas GK1.5 (anti-L3T4) and 53-6.72 (anti-Lyt-2). Supernatants from confluent cultures were filter sterilized and used at the dilutions indicated.

*Lymph node proliferation assay*

Seven days following immunization, the inguinal draining lymph nodes (DLN) were removed and single-cell suspensions prepared in RPMI-1640 medium (Gibco-BRL, Paisley, U.K.), supplemented with 5% foetal calf serum (FCS) (Flow Labs), glutamine at 2 mM (Gibco-BRL), penicillin at 100 IU/ml and streptomycin at 100 μg/ml (Gibco-BRL). In certain experiments, cells were preincubated with monoclonal antibodies prior to culture with proteins and peptides.

Cells were cultured at $5 \times 10^5$ cells/well in flat-bottomed 96-well microtitre plates (Nunc, Roskilde, Denmark). Cells were challenged, in triplicate culture wells, with peptide or proteins for 5 days. Proliferative responses were determined by measuring the incorporation of tritiated thymidine (0·5 μCi/well) (Amersham International, Amersham, U.K.) during the last 18 hr of culture. In some experiments, supernatants were removed 24 hr following stimulation with peptide or protein, for analysis of interleukin-2 (IL-2) production. Cells were harvested onto glass fibre filters (934-AH, Whatman, Maidstone, U.K.), using a Skatron cell harvester (LKB Wallac, Milton Keynes, U.K.) and counted in a Beckman LS 5000CE β-counter. Results were expressed as mean disintegrations per minute (d.p.m.), converted into stimulation indices (SI) as follows:

$$\text{Stimulation index} = \frac{\text{d.p.m. in stimulated cultures}}{\text{d.p.m. in control cultures}}$$

An SI of less than 2 was not considered significant.

*IL-2 assay*

IL-2 production by responding cells was measured 24 hr after stimulation with peptide or protein as previously described.[18] Supernatants (50 μl) were removed and incubated with $10^4$ cells of the IL-2-dependent CTLL line in a total volume of 100 μl for 24 hr. CTLL proliferation was determined by the incorporation of [$^3$H]thymidine (0·5 μCi/well) during the final 4 hr of culture. IL-2 values were expressed as Biological Response Modifiers Program (BRMP) U/ml as compared to a recombinant mouse IL-2 standard (Collaborative Biomedical Products, Bedford, MA).

## RESULTS

### Proliferative and IL-2 responses following immunization with V3:Ty-VLP

Four strains of mice (C3H/Hej, A.SW, BALB/c and C57BL/6) representing four haplotypes (H-$2^{k,s,d}$ and $^b$) were immunized with 50 μg of V3:Ty-VLP as an alum precipitate. Cells were then challenged in the DLN proliferation assay with IIIB V3 peptide at 10 μg/ml, rgp120 at 1 μg/ml, Ty protein at 1 μg/ml or control. Table 1 illustrates the proliferative responses obtained in the

**Table 1.** Proliferative responses (measured as stimulation indices) to IIIB V3 peptide, rgp120 and Ty protein following immunization of mice with 50 μg V3:Ty-VLP in alum

| | Stimulation indices ± SEM | | |
|---|---|---|---|
| Mouse strain (haplotype) | V3 peptide (10 μg/ml) | rgp120 (1 μg/ml) | Ty (1 μg/ml) |
| C3H/Hej (H-$2^k$) | 1·06 ± 0·06 | 0·87 ± 0·17 | 34·0 ± 9·2 |
| A.SW (H-$2^s$) | 2·3 ± 0·26 | 2·1 ± 0·08 | 39·5 ± 1·14 |
| BALB/c (H-$2^d$) | 17·2 ± 2·68 | 2·9 ± 0·63 | 275 ± 25·1 |
| C57BL/6 (H-$2^b$) | 51 ± 2·6 | 89 ± 4·8 | 249 ± 7·3 |

**Table 2.** IL-2 production (BRMP U/ml) by lymphocytes from mice immunized with V3:Ty-VLP and stimulated with IIIB V3 peptide, rgp120 or Ty protein for 24 hr *in vitro*

| | IL-2—BRMP units ± SEM | | | |
|---|---|---|---|---|
| | V3 peptide (10 μg/ml) | rgp120 (1 μg/ml) | Ty (1 μg/ml) | Control |
| C3H/Hej (H-2$^k$) | 0·356±0·009 | 0·026±0·001 | 0·665±0·073 | 0·024±0·001 |
| A.SW (H-2$^s$) | 0·334±0·013 | 0·241±0·016 | 11·60±1·62 | 0·015±0·006 |
| BALB/c (H-2$^d$) | 1·384±0·081 | 0·247±0·035 | 1·641±0·110 | 0·025±0·002 |
| C57BL/6 (H-2$^b$) | 2·556±0·267 | 2·202±0·313 | 11·26±1·04 | 0·038±0·067 |

four strains of mice. Background (control) responses were 143, 1616, 134 and 144 d.p.m. respectively.

Significant (SI >2) proliferative responses to IIIB V3 peptide and rgp120 were seen in three of the four haplotypes. C3H/Hej (H-2$^k$) mice did not respond to V3-IIIB or rgp120 although they showed a substantial response to the Ty protein. CBA mice, also of the H-2$^k$ haplotype, did show a low but significant response to V3 peptide and rgp120 in a subsequent experiment (SI of 3·7 and 2·6 respectively, see Fig. 3). Normal mouse DLN cells gave no proliferative responses to IIIB V3 peptide or rgp120 at the doses tested.

Supernatants from the same experiment were removed at 24 hr and tested against the IL-2-dependent CTLL line. The BRMP units obtained are shown in Table 2. The results essentially mirror the responses seen in the proliferation assay with one exception. The response of C3H-Hej to IIIB V3 peptide was significantly above background, indicating a low level of response to this peptide. Previous studies have shown that IL-2 production is a more sensitive indicator of Th-cell function,[11] and this, together with the responses seen in CBA mice (see Fig. 3) suggests that the H-2$^k$ haplotype is a low responder to IIIB V3 peptide. H-2$^s$ is also a low-responder haplotype.

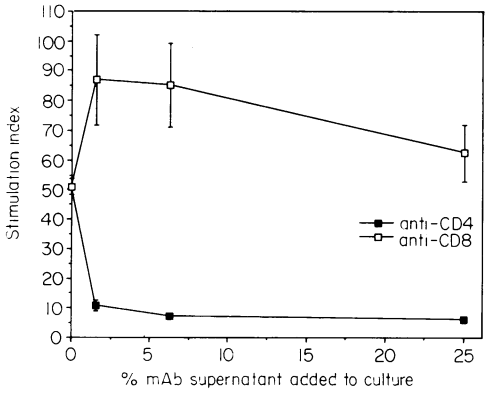


**Figure 1.** Suppression of proliferative responses to IIIB V3 peptide by anti-CD4 monoclonal antibody. Mice were immunized with 50 μg of V3:Ty-VLP and cells from DLN were challenged *in vitro* with V3 peptide at 10 μg/ml. Monoclonal antibodies were tested at 1/4, 1/16, 1/64 and 1/256.

### Effect of anti-CD4 and anti-CD8 monoclonal antibody (mAb) on responses to IIIB V3 peptide

To characterize the T-cell phenotype responding to IIIB V3 peptide, DLN cells from C57BL/6 mice immunized with V3:Ty-VLP were preincubated with anti-CD4 or anti-CD8 rat mAb for 1 hr prior to the addition of the peptide at 10 μg/ml. Figure 1 shows that the addition of the anti-CD4 mAb resulted in a dose-dependent reduction in the response to IIB V3 peptide. There was a 62·7% reduction in the response at 1/256 dilution of mAb. No such reduction was seen with anti-CD8 mAb. In BALB/c mice the SI was reduced from 17·2 to 1·6 following preincubation with anti-CD4 antibody at a dilution of 1/4 compared to a reduction to 12·5 with anti-CD8 mAb at a 1/4 dilution.

### The effect of V3:Ty-VLP immunization dose on proliferative responses to IIIB V3 peptide

BALB/c mice were immunized with V3:Ty-VLP at doses ranging from 0·3 to 175 μg. Cells from the DLN were challenged with IIIB V3 peptide at either 0·5 or 5·0 μg/ml. An immunization dose-dependent increase in proliferative response was observed (Fig. 2). Significant proliferative responses were seen following immunization with V3:Ty-VLP at 7, 35 and 175 μg. Back-

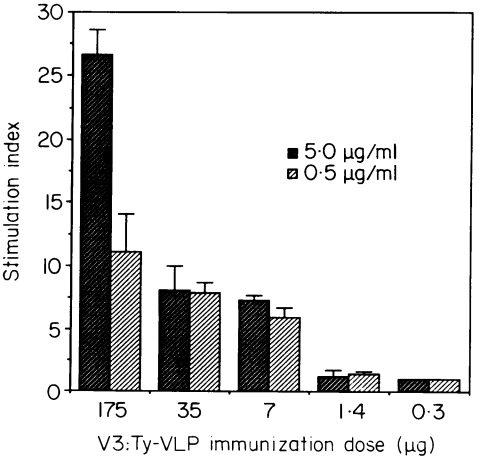


**Figure 2.** Proliferative responses to IIIB V3 peptide following immunization of BALB/c mice with V3:Ty-VLP at doses ranging from 0·3 to 175 μg. Cells from the DLN were challenged *in vitro* with V3 peptide at either 0·5 or 5·0 μg/ml.

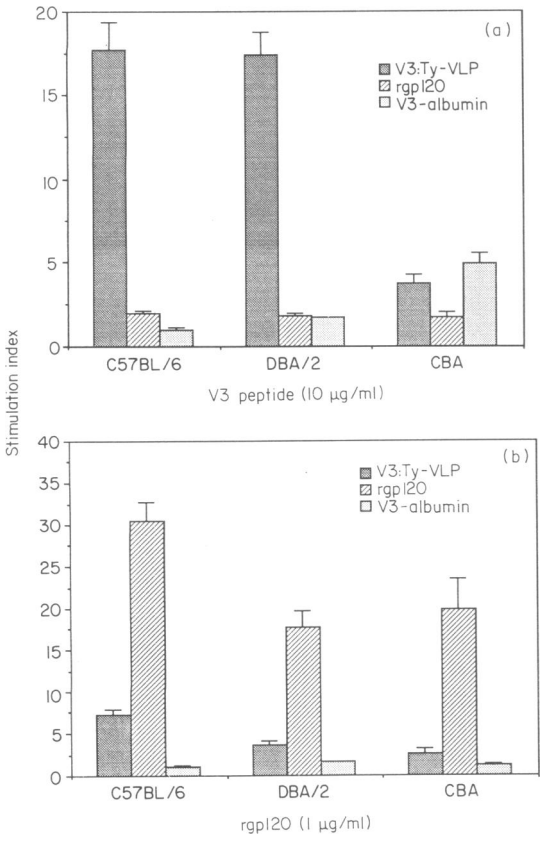


**Figure 3.** Proliferative responses to IIIB V3 peptide and rgp120 in three mouse haplocytes immunized with V3:Ty-VLP, rgp120 or V3-albumin. Mice were immunized with either 15 μg of V3:Ty-VLP, 17·3 μg of rgp120 or 11·7 μg of V3-albumin (equivalent to 1·575 μg of V3 sequence) as alum precipitates. Cells from the DLN were challenged *in vitro* with (a) 10 μg/ml of V3 peptide (HXB2) or (b) 1 μg/ml of rgp120 (BH10).

**Table 3.** Background proliferative responses (d.p.m.) in the three strains immunized with the three immunogens

| Immunogen | C57BL/6 | DBA/2 | CBA |
|---|---|---|---|
| V3:Ty-VLP | 2338 | 4050 | 1100 |
| rgp120 | 2658 | 13123 | 6530 |
| V3-alb | 670 | 410 | 198 |

**Table 4.** Proliferative responses to IIIB V3 peptide and MN V3 peptides following immunization with 50 μg of V3:Ty-VLP in alum, expressed as stimulation indices

| | Stimulation indices ± SEM in response to: | |
|---|---|---|
| Mouse strain | IIIB V3 peptide (10 μg/ml) | MN V3 peptide (10 μg/ml) |
| C3H-Hej (H-2$^k$) | 1·06 ± 0·06 | 1·1 ± 0·02 |
| A.SW (H-2$^s$) | 2·3 ± 0·26 | 3·7 ± 0·16 |
| BALB/c (H-2$^d$) | 17·2 ± 2·68 | 1·6 ± 0·16 |
| C57BL/6 (H-2$^b$) | 51·0 ± 2·62 | 56·0 ± 9·70 |

ground responses were 4585, 832, 639, 100 and 114 d.p.m. in the 175, 35, 7, 1·4 and 0·3 μg groups respectively.

### Enhanced proliferative responses following immunization with a particulate V3 immunogen

Having optimized immunization regimes and *in vitro* challenge conditions to demonstrate V3-specific proliferative responses, we then investigated the ability of V3:Ty-VLP to present V3 with the responses induced by rgp120 and V3-alb. Three strains of mice (C57BL/6, DBA/2 and CBA) were immunized with either 15 μg of V3:Ty-VLP, 17·3 μg of rgp120 or 11·7 μg of V3-alb. These represent immunization doses of 1·575 μg of V3 peptide sequence. After 7 days, cells from the DLN were challenged with IIIB V3 peptide at 10, 1·0, 0·1 or 0·01 μg/ml, or rgp120 at 1·0, 0·1 or 0·01 μg/ml. Figure 3 demonstrates that in C57BL/6 and DBA/2 mice, immunization with V3:Ty-VLP led to greater proliferative responses to IIIB V3 peptide (10 μg/ml) than those seen following immunization with rgp120 or V3-alb. Results showed the same trend at the lower IIIB V3 peptide doses but with diminishing SI values (data not shown). Responses to the V3 peptide in CBA (H-2$^k$) mice were generally low, and this is the case with C3H/Hej mice, another H-2$^k$ strain (see Table 2).

In contrast to the V3-specific proliferation data, responses to *in vitro* challenge with rgp120 (1μg/ml) were higher in mice immunized with rgp120 than with V3:Ty-VLP. However, immunization with V3:Ty-VLP led to significant responses to rgp120 in all three strains of mice, with the CBA strain again being the poorest responder. V3-alb failed to prime for gp120-induced proliferation. Similar trends were seen with other challenge doses of rgp120 (data not shown).

Background proliferative responses in the three strains immunized with the three immunogens were as in Table 3. The reason for high background responses in two groups of mice immunized with rgp120 is not known.

### Proliferative responses to IIIB V3 and MN V3 peptide following V3:Ty-VLP immunization

Four mouse haplotypes (C3H-Hej, A.SW, BALB/c and C57BL/6) were immunized with 50 μg of V3:Ty-VLP as an aluminium hydroxide precipitate. Cells from the DLN were removed and challenged *in vitro* with V3 peptide (HXB2) and MN V3 peptides at 10 μg/ml. Proliferative responses were seen to both peptides in the A.SW and C57BL/6 mice as shown in Table 4. The results imply that H-2$^b$ (C57BL/6) and H-2$^s$ (A.SW) mice are responding to a different epitope within the 40mer V3 sequence than are the H-2$^d$ mice.

### The effects of adjuvant on the immunogenicity of V3:Ty-VLP

Having demonstrated the ability of V3:Ty-VLP with alum adjuvant to induce proliferative responses, we then assessed the immunogenicity of V3:Ty-VLP in the absence of adjuvant. C57BL/6 mice were immunized with 50 μg of V3:Ty-VLP with or without alum adjuvant and DLN cells were stimulated *in vitro* with IIIB V3 peptide at 0·01–10 μg/ml. Figure 4 demonstrates that, even without adjuvant, V3 peptide-specific proliferative responses are observed, with stimulation indices as high as 7·5. Responses were higher when V3:Ty-VLP were given with

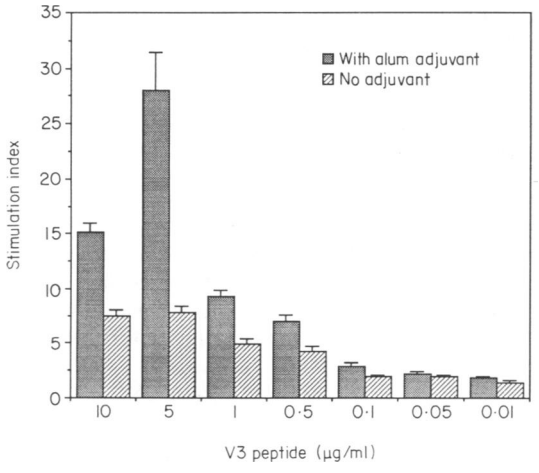


**Figure 4.** Proliferative responses to IIIB V3 peptide following immunization of C57BL/6 mice with 50 μg of V3:Ty-VLP with or without adjuvant. Cells from the DLN were challenged *in vitro* with V3 peptide at doses ranging from 0·01 to 10 μg/ml.

alum. The background proliferative responses were 616 d.p.m. in the non-adjuvant group and 1955 d.p.m. in the alum adjuvant group. Similar results were obtained with BALB/c mice, and V3-alb was not able to prime for V3-specific proliferative responses in the absence of adjuvant (data not shown).

## DISCUSSION

Following immunization of mice with V3:Ty-VLP, proliferative T-lymphocyte responses were detected *in vitro* against the V3 sequence of HIV-1. The responses were induced using V3:Ty-VLP either in conjunction with aluminium hydroxide or without adjuvant, although responses were lower in the absence of adjuvant. These observations are of significance because aluminium hydroxide is the only adjuvant currently licensed for human use. Good responses were seen using lymphocytes from mice of two different haplotypes (H-$2^b$ and H-$2^d$) and weak responses were observed in H-$2^k$ and H-$2^s$ mice. Since a vaccine would ideally need to induce immunity in a wide range of human HLA haplotypes, the results from these four haplotypes are encouraging. The responding cells have been identified as $CD4^+$ Th lymphocytes and were characterized by IL-2 production in response to antigen and the abrogation of the response by anti-CD4 antibody.

Lymphocytes from mice immunized with V3:Ty-VLP proliferated in response to *in vitro* stimulation with rgp120. Although the precise conformation and glycosylation pattern of insect-cell derived gp120 is not identical to that of native viral gp120,[19] the primary amino structure, from which T-cell epitopes are derived, will be unchanged. It is, therefore, highly likely that V3-specific T lymphocytes, primed following immunization with V3:Ty-VLP will recognize native gp120. Clearly, the induction of memory T lymphocytes, able to respond to a subsequent challenge with HIV, would be an essential component of an HIV vaccine.

In order to evaluate the comparative efficiency of V3:Ty-VLP in the induction of proliferative responses we also analysed the responses of two non-particulate immunogens. Recombinant gp160 and 120 are currently being evaluated by several groups as components of an HIV vaccine[6,20,21] and the conjugation of peptides to a larger carrier protein is a conventional method for enhancing immunogenicity.[16] In the comparative experiments, V3-specific proliferative responses were consistently higher following immunization with V3:Ty-VLP than with recombinant gp120 or V3-albumin. It is possible that the lower V3-specific responses induced by rgp120 are attributable to the single amino acid change (R⇒S) within the V3 loop sequences of IIIB V3 peptide and rgp120 (BH10). However three T-cell epitopes have been defined in humans which do not include this residue.[10,11] A fourth T-cell epitope has been defined within the V3 loop following immunization of goats with a BH10-based peptide containing a serine residue at this position.[22] However, the effects of single amino acid changes on T-cell recognition were not investigated. Finally, the cross-reactivity with MN V3 peptide observed in the H-$2^b$ mice suggests that the amino acid change is not critical. Overall, the data indicate that immunogenicity of the V3 loop is enhanced when the sequence is presented as a Ty-VLP.

The ability of V3:Ty-VLP to prime T cells to recognize IIIB and MN V3 peptides in H-$2^b$ and H-$2^s$ mice but not in H-$2^d$ and H-$2^k$ suggests that two different epitopes are being recognized. Although we have not defined the epitopes involved, it is of interest to note that Hale *et al.*[23] described an epitope, HP19, within this region and found strong responses in H-$2^b$ mice and weak responses in H-$2^k$. They did not include H-$2^d$ or H-$2^s$ mice in their study. However, T-cell epitopes which are shared between isolates will be useful in the development of vaccines.

The mechanism responsible for the enhanced immunogenicity, as measured by Th-cell responses, remains to be elucidated. However, the comparative data suggest that the particulate nature of the Ty-VLP is important, as the V3:Ty-VLP were more effective than the two non-particulate immunogens. This suggestion is supported by evidence from other particulate antigen presentation systems. For example, ISCOMS (immunostimulatory complexes), containing peptides or proteins in stable 35-nm diameter matrices of the adjuvant Quil A, have been shown to be up to 10 times more immunogenic than the same viral peptide or protein in a micelle structure alone.[24] Similarly, liposomes have been used to entrap antigens in a lipid shell structure of between 20 nm and 1 μm, with improved immunogenicity being demonstrated using a wide variety of antigens.[25] In addition epitope sequences can be inserted into the core antigen of hepatitis B virus (HBcAg), yielding particles which augment the immune response to the inserted sequence.[26-28] The amelioration of immune responses by particulate antigen presentation systems may be due to a number of factors. These include the polymeric nature of the particles, increased activation and uptake by antigen-presenting cells, and increased longevity of the particle *in vivo*. The immune system is adapted to dealing with particulate pathogens, such as bacteria or viruses. Immunization with Ty-VLP and other particulate immunogens may therefore lead to the uptake, processing and presentation of epitopes more effectively than immunization with non-particulate proteins. Indirect evidence for this hypothesis may be that the only recombinant DNA subunit vaccines sufficiently efficacious to be licensed for human use are hepatitis B surface antigen preparations, which also assemble into particulate structures.[29]

The proliferative responses to *in vitro* stimulation with

rgp120 are stronger following immunization with rgp120 than those following immunization with V3:Ty-VLP. This difference can, almost certainly, be attributed to the presence of at least two additional T-cell epitopes which have been described on gp120, noteably T1 (aa 428-443) and T2 (aa 112-124).[23] This particular comparison is, therefore, weighted heavily against the V3:Ty-VLP, which contain only the V3 epitope. However, these results suggest that the addition of further T-cell epitopes will lead to a further enhancement of proliferative responses, as described for synthetic peptide approaches.[22,30] The Ty-VLP technology is ideally suited to a multiple epitope approach, being able to tolerate a wide range of additional protein sequence without disrupting particle formation.[31] An attractive approach to vaccine design may be to combine T- and B-cell epitopes within a single VLP construction to produce a particulate, polyvalent and multivalent immunogen.

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