

Induction of High-Titer Neutralizing Antibodies, Using Hybrid Human Immunodeficiency Virus V3-Ty Viruslike Particles in a Clinically Relevant Adjuvant

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The localization of neutralization determinants within the envelope glycoproteins of human immunodeficiency virus (HIV) has been largely achieved by immunizing small animals in conjunction with Freund's adjuvant. However, for eventual use in humans, candidate HIV vaccine components must also be efficacious in a nontoxic formulation. We describe here the production of hybrid Ty viruslike particles carrying the major neutralizing domain of HIV and demonstrate the induction of high-titer virus-neutralizing antibodies and an HIV-specific T-cell proliferative response after immunization in conjunction with aluminum hydroxide. As aluminum hydroxide and aluminum phosphate are the only adjuvants currently licensed for use in humans, these observations have implications for the development of an effective vaccine against HIV.

The humoral immune response can be an important component of protective immunity against infectious agents, and one strategy for immunoprophylaxis against AIDS may be the administration of an immunogen designed to elicit virus-neutralizing antibodies. Although many laboratories have demonstrated that all or parts of the human immunodeficiency virus (HIV) envelope glycoproteins induce neutralizing antibodies when administered in Freund's adjuvant (9, 18, 22, 33, 34, 36), very few studies have been done using clinically approved adjuvants. Aluminum hydroxide has generally only been used in studies involving primates, and in most cases in which neutralization has been observed, the titers have been low (3, 4, 6, 33). However, significant progress toward the development of an HIV vaccine has been made recently with the demonstration that recombinant gp120 formulated in aluminum hydroxide can elicit protective immunity in chimpanzees against a homologous strain of HIV type 1 (HIV-1) (5). The protection was correlated with the induction of high-titer neutralizing antibodies and also with the presence of antibodies targeted against the principal neutralizing determinant of HIV (19) contained within the disulfide-cross-linked third variable domain (V3 loop) of gp120. Although no adverse side effects were observed in the immunized chimpanzees, the immunosuppressive effects that may result from gp120 binding to CD4 and inhibiting T-cell function in humans should still be taken into consideration (10, 13, 24, 31). The use of immunogens containing neutralization determinants but deficient in CD4 binding may therefore be advantageous. Surprisingly, despite consider-

able interest in the ability of V3 loop peptides to elicit neutralizing antibodies (19, 34, 35, 38) and the *in vivo* evidence that anti-V3 antibodies may prevent infection by HIV (5, 11), there have been no reports of the efficacy of V3-based immunogens when administered in adjuvants approved for use in humans.

The approach that we took was to analyze the immunogenicity of a V3 loop sequence presented as a particulate fusion protein. We have shown previously that a protein encoded by the *Saccharomyces cerevisiae* retrotransposon Ty can be used as a carrier for producing recombinant HIV antigens that induce both B-cell and T-cell responses in experimental animals (1, 30). The Ty particle itself can tolerate a wide range of additional protein sequence without disruption, allowing small peptide-sized fragments, protein domains, and full-length proteins to be accommodated (1, 15, 30). In addition, any hybrid Ty viruslike particle (Ty-VLP) can be purified by using a generic protocol based on the physical properties of the particle. To produce hybrid HIV V3-Ty-VLPs containing a V3 loop, synthetic oligomers encoding gp120 amino acids 295 to 333 from isolate HXB2 (14) were inserted into the *Bam*HI site of plasmid pMA5620 (1) (Fig. 1a). The resulting plasmid (pOGS514) therefore contained a Ty-HIV fusion gene under the control of the efficient yeast phosphoglycerate kinase promoter (Fig. 1a). After transformation of plasmid pOGS514 into yeast cells, the fusion protein was expressed at high levels and assembled into 50-nm particles (Fig. 1b). These hybrid HIV V3-Ty-VLPs were purified by a standard procedure that has been described previously (15), and the presence of the V3 sequence was confirmed by Western immunoblotting (data not shown). The location of the HIV component was analyzed by immunogold electron microscopy with a V3 loop-specific monoclonal antibody (MAb) (Fig. 1c). When control

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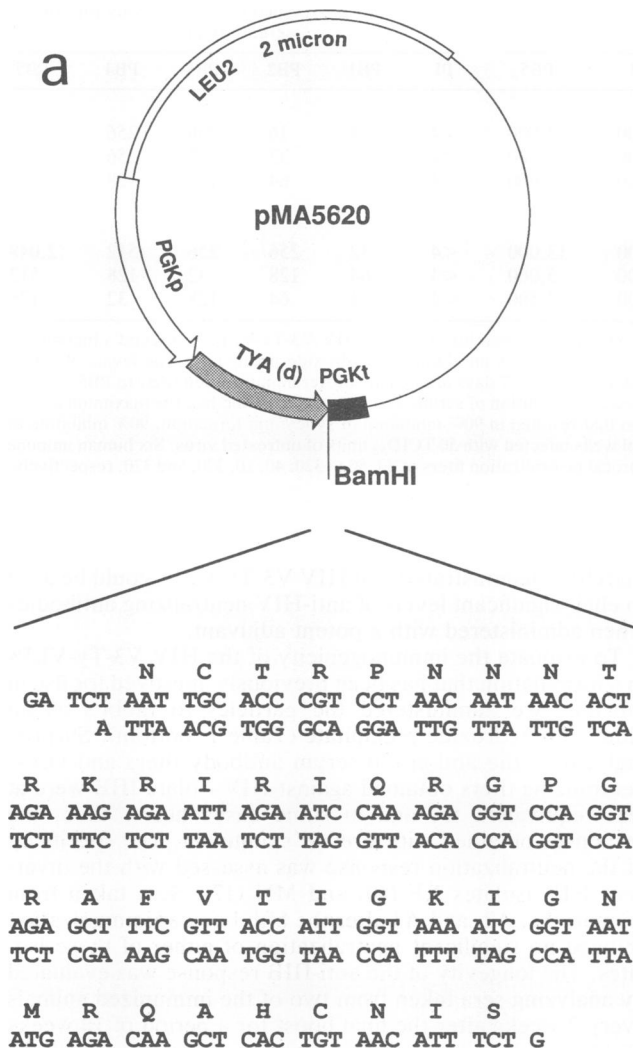


FIG. 1. Construction of HIV V3-Ty-VLPs. (a) Structure of the expression vector pMA5620 and the nucleic acid and predicted amino acid sequence of oligomers encoding the V3 loop of HIV isolate HXB2. Plasmid pMA5620 has been described previously (1) and contains the first 381 codons of the *TYA* gene from the yeast retrotransposon Ty1-15 (20) [*TYA(d)*; shaded arrow], promoter sequences from the yeast *PGK* gene (open arrow), a *LEU2:2μ* selection and replication module (open box), a replication and ampicillin selection module from pBR322 (thin line), and a *PGK* terminator fragment containing stop codons in all three reading frames and a transcriptional stop signal (black box). The codons of the synthesized oligomers were assigned according to yeast codon bias, using published tables of codon usage (16). Annealed oligomers were inserted into the *Bam*HI site of pMA5620 to generate plasmid pOGS514. (b) Electron micrograph of HIV V3-Ty-VLPs, prepared for electron microscopy by procedures described previously (28). (c) Electron micrograph of HIV V3-Ty-VLPs probed with a gold-labeled anti-gp120 MAb. Immunogold labeling was done by incubating HIV V3-Ty-VLPs with a gp120-specific MAb, 9284 (36) (Du Pont; raised against isolate IIIB). Bound antibody was detected after incubation with anti-mouse antibody conjugated to 5-nm gold particles (Sigma) and staining with 3% phosphotungstic acid.

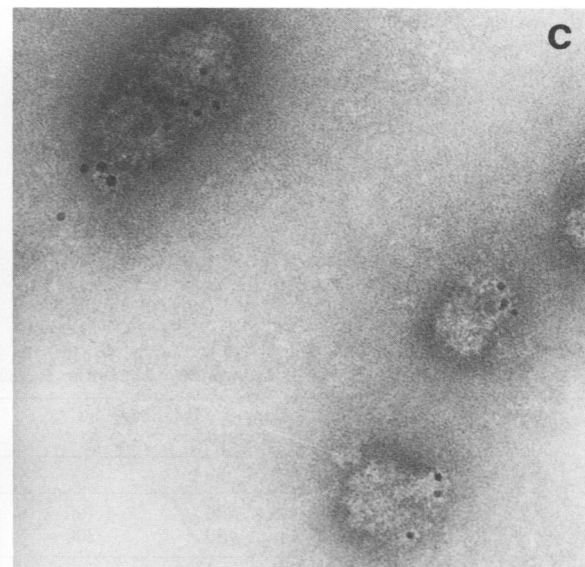
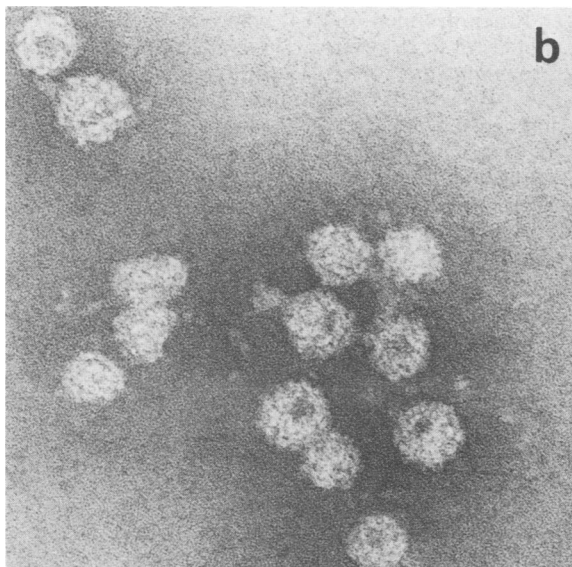


TABLE 1. Serum antibody and neutralizing antibody titers of rabbits immunized with HIV V3-Ty-VLPs^a

Adjuvant and animal no.	Reciprocal serum antibody midpoint titer of serum sample:						Reciprocal HIV neutralizing antibody titer of serum sample:					
	PI	PB1	PB2	PB3	PB4	PB5	PI	PB1	PB2	PB3	PB4	PB5
Freund's adjuvant												
F1	<100	200	1,200	11,000	13,000	13,000	<4	4	16	256	256	512
F2	<100	200	2,000	8,000	9,000	2,000	<4	8	32	32	256	128
F3	<100	150	900	9,000	12,500	5,000	<4	256	64	128	128	512
Aluminum hydroxide												
A1	<100	2,000	1,000	6,500	9,000	13,000	<4	32	256	256	512	2,048
A2	<100	900	400	500	2,000	5,000	<4	64	128	32	128	512
A3	<100	<100	2,000	4,500	6,000	2,500	<4	<4	64	128	32	128

^a Animals F1 to F3 received a priming immunization in Freund's complete adjuvant and were then boosted with HIV V3-Ty-VLPs in Freund's incomplete adjuvant. Animals A1 to A3 received priming and booster immunizations of HIV V3-Ty-VLPs as an aluminum hydroxide precipitate. The levels of serum antibodies and virus-neutralizing antibodies were assessed in preimmune sera (PI) and in sera taken 7 days after each booster immunization (PB1 to PB5). Serum antibodies against gp120 were measured by ELISA, and midpoint titers were calculated as the dilution of serum which gave rise to one-half the maximum optical density at 450 nm. Neutralizing antibody titers are expressed as the dilution of serum that resulted in 90% inhibition of syncytium formation. 90% inhibition of syncytium formation was defined as the degree of cytopathic effect observed in control wells infected with 50 TCID₅₀ units of untreated virus. Six human immune sera (QC1 to QC6) from anonymous British HIV-positive blood donors (27) gave reciprocal neutralization titers of 80, 80 to 320, 40, 10, 320, and 320, respectively, in this assay.

Ty-VLPs were probed with the same MAb, no specific labeling was observed. As the interior of the particles is unlikely to be accessible to the gold-antibody complexes, the specific labeling of the HIV V3-Ty-VLPs indicates that at least part of the V3 loop region is exposed on the surface of the particles.

To establish that the V3 loop region was immunogenic when presented in this conformation, we initially used purified particles to immunize rabbits in conjunction with Freund's adjuvant. Rabbits were immunized intramuscularly with 500 µg of purified HIV V3-Ty-VLPs at 2-week intervals. A 500-µg sample of HIV V3-Ty-VLPs contains 50 µg of the HIV V3 loop component. The animals received a priming immunization in Freund's complete adjuvant and were then boosted with HIV V3-Ty-VLPs in Freund's incomplete adjuvant. Serum antibody titers were determined against recombinant gp120 (isolate IIIB [37]) secreted from Chinese hamster ovary (CHO) cells (Celltech Ltd.; obtained from the MRC AIDS Directed Programme) in an enzyme-linked immunosorbent assay (ELISA). Neutralization titers were measured by an infection inhibition assay based on the method described by Kinney-Thomas et al. (21). Briefly, heat-inactivated serum was incubated with 500 50% tissue culture infectious dose (TCID₅₀) units of HIV-1 isolate IIIB for 1 h at 37°C and then added to 10⁵ C8166 cells. After 48 h at 37°C, the cultures were examined for the presence of syncytia. Titers were expressed as the dilution of serum that resulted in 90% inhibition of syncytium formation. Table 1 (top) demonstrates that both serum antibodies and virus-neutralizing antibodies were detected after a single boost and increased to high titers after subsequent boosts. These data

therefore demonstrated that HIV V3-Ty-VLPs could be used to elicit significant levels of anti-HIV neutralizing antibodies when administered with a potent adjuvant.

To evaluate the immunogenicity of the HIV V3-Ty-VLPs in a formulation that has been previously approved for use in humans, we administered the particles to rabbits as an aluminum hydroxide precipitate (Table 1, bottom). Surprisingly, both the anti-gp120 serum antibody titers and virus-neutralizing titers obtained against HIV isolate IIIB were at least equivalent to, and in some cases higher than, the responses obtained with Freund's adjuvant. The specificity of the neutralization response was assessed with the divergent HIV isolates RF (40) and MN (17). Sera taken from animals A1, A2, and A3 after the fifth booster immunization showed no significant neutralization of either of these isolates. The longevity of the anti-IIIB response was evaluated by analyzing sera taken from two of the immunized animals every 2 weeks after the final boost for a period of 10 weeks (Table 2). The neutralizing antibody titers of animal A1 remained constant at 1/512 for 8 weeks after the final boost and then dropped to 1/64, whereas the ability of sera from animal A3 to neutralize HIV isolate IIIB declined gradually throughout the 10-week period, with a half-life of 20 to 30 days. However, in both animals, significant levels of serum and neutralizing antibodies were still present 10 weeks after the last immunization.

Javaherian et al. (19) and Meloen et al. (29) have recently demonstrated that the major neutralizing epitope of HIV-1 isolate IIIB is defined by eight amino acids (QRGPGRAF) at the tip of the V3 loop (Fig. 2A). We therefore investigated whether the antibodies raised against the HIV V3-Ty-VLPs

TABLE 2. Longevity of the antibody response in animals immunized in conjunction with aluminum hydroxide^a

Animal no.	Reciprocal serum antibody midpoint titer at wk:					Reciprocal HIV neutralizing antibody titer at wk:				
	2	4	6	8	10	2	4	6	8	10
A1	1,000	4,000	800	1,100	300	512	512	512	512	64
A3	200	300	400	200	300	64	64	32	16	16

^a The levels of serum antibodies and neutralizing antibodies were assayed in sera taken from animals at 2-week intervals after the final immunization with HIV V3-Ty-VLPs.

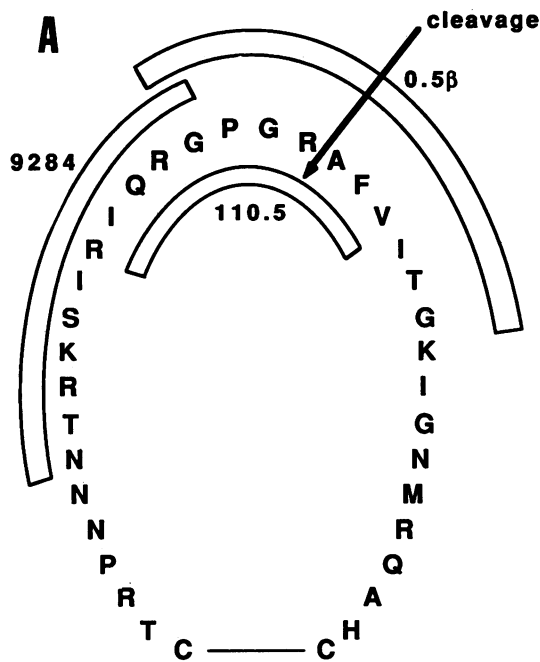
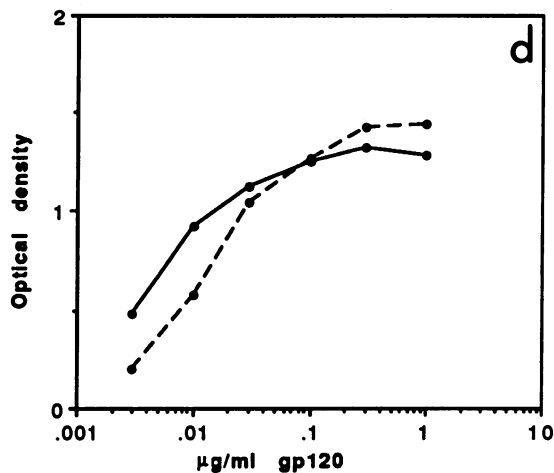
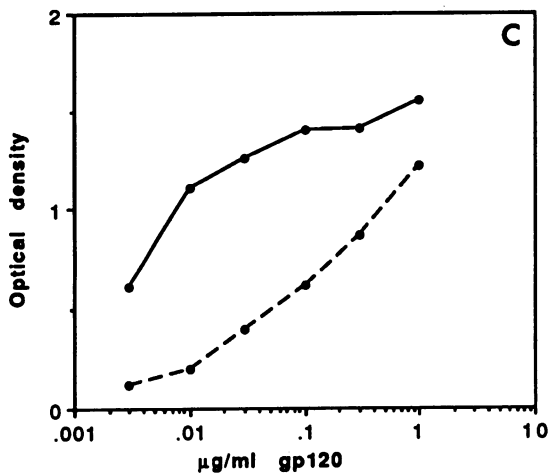
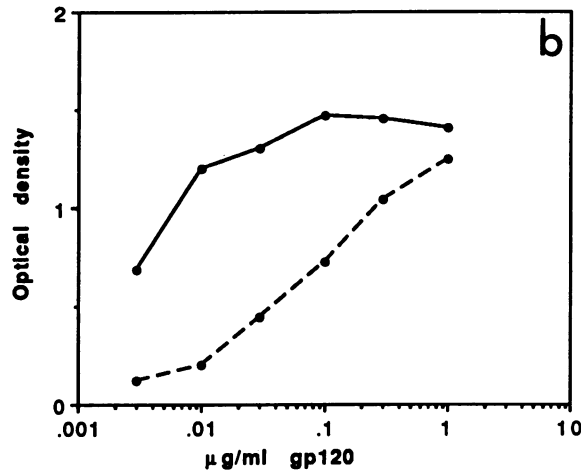
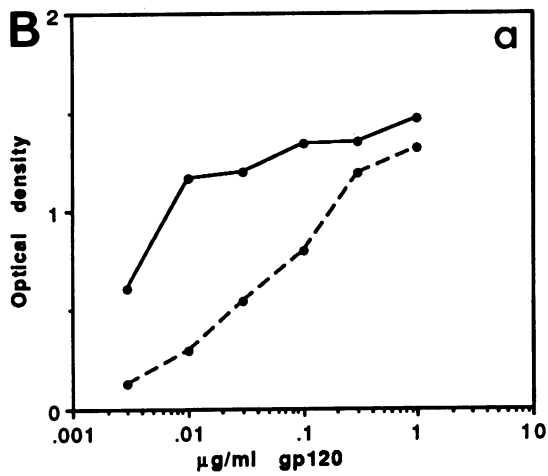


FIG. 2. Binding of rabbit serum and gp120-specific MAbs to intact and cleaved recombinant gp120. (A) Schematic representation of the epitopes recognized by V3 loop-specific MAbs 9284 (39) (Du Pont), 110.5 (23), and 0.5 β (25). The loop sequence shown is from HIV-1 isolate IIIB. CHO-derived recombinant gp120 (rgp120; Celltech Ltd.; obtained from the MRC AIDS Directed Programme) can be specifically cleaved within the V3 loop by extended culture of the CHO cells before harvesting and purification of the material (41). The cleavage site within the V3 loop is shown. (B) Binding profiles of rabbit serum A1 and gp120-specific MAbs to intact and cleaved rgp120. Intact rgp120 (solid lines) or 95%-cleaved rgp120 (broken lines) at various concentrations was captured indirectly onto microtiter wells by a polyclonal sheep antibody raised against a C-terminal peptide of gp120 (D7324; Aalto BioReagents, Dublin, Eire) (32). The bound rgp120 was then reacted with a fixed concentration of serum from rabbit A1 taken 7 days after the fifth boost (1/3,000) (a), MAb 110.5 (1/100,000 dilution of ascites fluid) (b), MAb 0.5 β (1/100,000 dilution of ascites fluid) (c), MAb 9284 (0.1 μ g/ml) (d). Bound antibody was detected after incubation with alkaline phosphatase-conjugated anti-species antibodies (Dakopatts, High Wycombe, United Kingdom and development with the AMPAK ELISA amplification system (Novo Biolabs, Cambridge, United Kingdom).



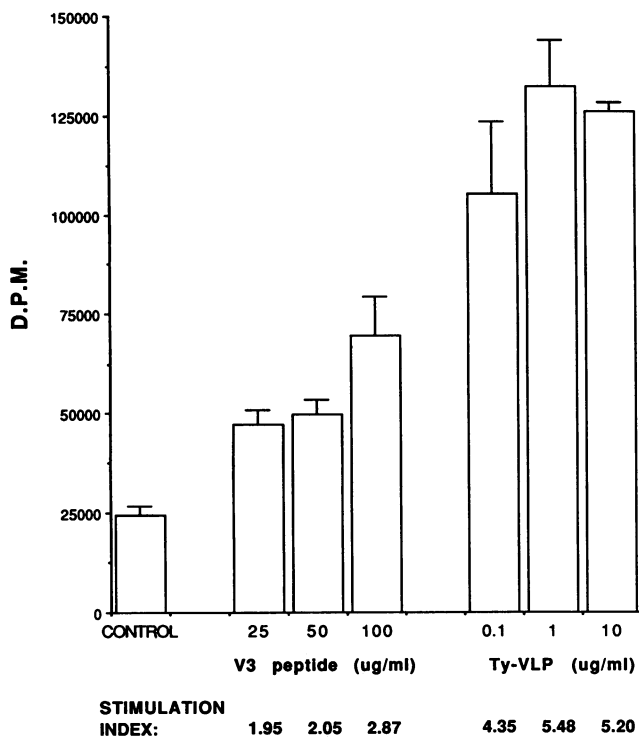


FIG. 3. Proliferative responses of immune mouse cells after in vitro stimulation with a V3 loop-specific synthetic peptide or control Ty-VLPs. C3H-HE mice were immunized with 50 μ g of HIV V3-Ty-VLPs as an aluminum hydroxide precipitate. Inguinal and periaortic lymph nodes were aseptically removed after 10 days, and a single-cell suspension was prepared. V3 loop peptide (Cambridge Research Biochemicals, Cambridge, United Kingdom) and control Ty-VLPs were added to aliquots of cells to give final concentrations as shown. Control wells contained cells with no peptide or Ty-VLPs added. After 96 h of incubation, cultures were pulsed with 0.5 μ Ci of [3 H]thymidine (Amersham) for 18 h. [3 H]thymidine incorporation was measured by liquid scintillation spectroscopy. The bars represent the mean of three replicates, with the standard error presented as a vertical line. The stimulation indices were calculated by dividing the mean of the test wells by the mean of the control wells.

contained a significant number of molecules that bound to this region. The ability of serum from animal A1 to bind intact recombinant gp120 and gp120 that had been proteolytically cleaved at a single site within the V3 loop (41) (Fig. 2A) was compared with the binding of three characterized gp120-specific MAbs. The binding profiles of the rabbit A1 serum, MAb 110.5 (23), and MAb 0.5 β (25) were comparable, and binding was reduced when the epitope at the tip of the loop was destroyed by cleavage (Fig. 2B). In contrast, the binding of MAb 9284 (39) was unaffected by cleavage of gp120. It has been shown previously that MAbs 110.5 and 0.5 β bind to sequences that include the tip of the loop (26, 39), whereas MAb 9284 binds an amino acid sequence on the left-hand side that does not overlap the Arg-Ala cleavage site (39) (Fig. 2A). The binding profile of the anti-HIV V3-Ty-VLP serum is therefore consistent with a significant proportion of the antibodies being targeted to the major neutralizing epitope as delineated by Javaherian et al. (19) and Meloen et al. (29).

Traditionally, the success of vaccines against other pathogens has depended on the ability to induce immunological

memory such that anamnestic responses are achieved after exposure to the invading organism. This requires the generation of antigen-specific memory cells which in turn requires the involvement of T-helper cells. Wahren et al. (42) have recently shown that peripheral lymphocytes from HIV-1-positive individuals proliferated in response to peptides containing sequences from the V3 loop of isolate BRU. In addition, Palker et al. (35) have demonstrated V3 loop-specific T-cell proliferative responses after immunization of goats with synthetic peptides in conjunction with Freund's adjuvant. We therefore investigated whether the HIV V3-Ty-VLPs could prime T-helper-cell populations when administered in aluminum hydroxide. A significant proliferative response was obtained after in vitro stimulation of immune mouse cells with a peptide containing the V3 loop sequence of HIV-1 isolate HXB2 (Fig. 3), demonstrating that HIV V3-Ty-VLPs can elicit the generation of HIV-specific T-helper-cell populations. To determine whether antigen-specific memory cells had been generated in vivo, we administered an additional boost of HIV V3-Ty-VLPs to rabbit A3 after a 6-month rest. At the time of the boost immunization, no neutralizing antibodies were detected. However, sera taken 7, 14, and 21 days after immunization had neutralizing antibody titers of 1/16, 1/32, and 1/128, respectively, indicating rapid recall of the antibody response to the V3 loop.

The demonstration that high-titer neutralizing antibodies can be induced by using hybrid HIV V3-Ty-VLPs in a formulation relevant for use in humans constitutes an important step toward the development of an effective vaccine against HIV. However, there are still several obstacles to be overcome. First, it will be necessary to demonstrate that high levels of neutralizing antibodies can be sustained. In primate studies, neutralizing antibody titers declined to basal levels within 10 weeks (2-4, 6, 33). Although it is difficult to make comparisons between species, significant neutralizing antibodies were still present in rabbits immunized with hybrid HIV V3-Ty-VLPs 10 weeks after the final boost. However, for an HIV vaccine to be efficacious in humans, it may be necessary to maintain high levels of antibodies for several months. Second, if the levels of neutralizing antibodies gradually decline, a rapid recall of the response must be demonstrated after exposure to the virus. Although boosting with V3-Ty-VLPs resulted in recall of the antibody response to the V3 loop, it is possible that both Ty-specific and V3-specific T-helper cells were involved. In a natural infection, only HIV-specific responses will be of relevance, and it may therefore be necessary to increase the repertoire of HIV-specific T-cell epitopes in the vaccine formulation. Third, any vaccine candidate based on gp120 must take into account the sequence variability observed between isolates and the type specificity of the antibody response to the V3 loop determinant. One approach to overcome these problems might be to use a mixture of antigens containing V3 loop regions from the most frequently occurring isolates (12) in conjunction with additional T-helper-cell epitopes (7, 8) and specific immune enhancers such as T- or B-cell-stimulating cytokines. The ease with which such sequences can be accommodated by Ty-VLPs may make this an attractive route to the eventual production of a particulate combination HIV vaccine.

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