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Induction of neutralizing antibodies and cytotoxic T lymphocytes in Balb/c mice immunized with virus-like particles presenting a gp120 molecule from a HIV-1 isolate of clade A

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Abstract

We have recently developed a candidate HIV-1 vaccine based on virus-like particles (VLPs) expressing a gp120 from an Ugandan HIV-1 isolate of the clade A (HIV-VLP_As). In vivo immunogenicity experiments were performed in Balb/c mice, with an immunization schedule based on a multiple-dose regimen of HIV-VLP_As without adjuvants, showing a significant induction of both humoral and cellular immunity. The Env-specific cellular response was investigated in vitro, scoring for both the proliferative response of T helper cells and the cytolytic activity of cytotoxic T lymphocytes (CTLs). Furthermore, immune sera showed $>50\%$ neutralization activity against both the autologous field isolate and the heterologous T cell adapted B-clade HIV-1_{IIIB} viral strain. This is one of the first examples of HIV-1 vaccines based on antigens derived from the A clade, which represents $>25\%$ of all isolates identified world wide. In particular, the A clade is predominant in sub-Saharan countries, where 70% of the global HIV-1 infections occur, and where vaccination is the only rational strategy for an affordable prevention against HIV-1 infection.

Keywords: Vaccine; HIV-1; Clade A; Virus-like particles

1. Introduction

The development of an effective, safe and affordable vaccine strategy represents a crucial goal for both industrialized and developing countries.

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In fact, although the highly active antiretroviral therapy (HAART) induces a dramatic reduction in both HIV-related morbidity and mortality, it fails to eradicate the viral infection and selects resistant viral populations which may not be controllable with alternative and effective 'salvage' strategies (Palella et al., 1998; Fatkenheuer et al., 1999; Vandamme et al., 1999; Falloon, 2000).

The role of neutralizing antibodies (NA) in the containment of HIV-1 infection is still controversial. In primate studies, high levels of NA, either induced by vaccination protocols or passively infused, have been correlated with a protective immune response (Bruck et al., 1994; Girard et al., 1995; Mascola et al., 1999; Baba et al., 2000) on the contrary, a late and weak neutralizing activity of anti-HIV-1 antibodies has been observed in HIV-1-infected individuals (Moog et al., 1997). These low levels and the possible modest role of NA in vivo could be due to the early HIV-related immune impairment; in long-term non-progressors and in non-transmitting mothers, however, a protective role for NA has been surmised (Scarlatti et al., 1993; Cao et al., 1995; Montefiori et al., 1996).

CD8 + T lymphocytes (CTLs) have been reported to be critical for the control of the HIV-1 infection progression, considering that the early containment of HIV-1 replication coincides temporarily with the appearance of a virus-specific CTL response (Pantaleo et al., 1994; Koup et al., 1994); furthermore, in the long-term non-progressor HIV-1-infected group, a high-frequency CTL response is correlated with the control of virus load and a stable clinical status (Musey et al., 1997; Ogg et al., 1998). Therefore, an effective preventive HIV-1 vaccine should elicit both virus-specific neutralizing antibodies as well as CTLs, which, although sporadically detected, could provide a supply of memory T cells able to expand to effector CTLs more rapidly and in larger numbers than would do naive T cells after a HIV-1 infection (Letvin, 1998).

In this respect we have chosen a vaccine approach based on virus-like particles (VLPs) characterized by the capacity of inducing a strong humoral as well as cellular immune response, without risks of pathogenicity. The HIV-1 Pr55gag precursor protein, when expressed in recombinant baculovirus-infected cells, assembles as immature, non-replicating and non-infectious HIV-VLPs (Gheysen et al., 1989; Wagner et al., 1994). The Gag-based VLP model has been employed to deliver additional antigenic structures, such as whole proteins or specific individual epitopes, with an effective induction of both arms of the immune response (Haffar et al., 1991; Rovinski et al., 1992; Griffiths et al., 1993; Wagner et al., 1994; Osterrieder et al., 1995; Deml et al., 1997; Tobin et al., 1997; Montefiori et al., 2001). Different approaches have been pursued to insert HIV-1 envelope epitopes into the Gag protein (chimeric VLPs), in order to induce NA. In particular, HIV-1 envelope V3 epitopes have been introduced into dispensable Pr55gag domains (Griffiths et al., 1993; Wagner et al., 1994) or fused to the 3′ end of the *gag* ORF, taking advantage of the ribosomal frameshifting signals (Tobin et al., 1997). Both strategies have resulted in the induction of an efficient immune response against the HIV-1 epitopes, although the antibody response against the Gag protein appeared much stronger than against the envelope epitopes (Wagner et al., 1996; Tobin et al., 1997). In order to improve the Env antigenicity and to present conformational epitopes, the entire gp120 molecule has been expressed on the Pr55gag-VLPs and anchored through the trans-membrane (TM) portion of the Epstein-Barr virus (EBV) gp220/350 (Env–Gag–hybrid VLPs). This approach increases the expression and stability of the gp120 glycoprotein on the VLPs surface, without affecting its oligomerization (Deml et al., 1997; Buonaguro et al., 2001), and results in a relevant Env-specific immune response.

The gp120 glycoprotein selected for these HIV-VLPs was derived from an Ugandan HIV-1 isolate of the A clade (UG5.94UG018), previously characterized in our laboratory, which shows an average homology of 87.4% with A clade isolates (Buonaguro et al., 1995, 1998b). In addition to the Pr55gag coding region, Pol and Nef epitopes have been introduced in the resulting HIV-VLP$_A$s for subsequent further analysis (Buonaguro et al., 1998a, 2001).

The development of a candidate preventive vaccine targeted to a non-B gp120 envelope molecule,

has been prompted by the large excess of non-B isolates circulating in developing countries, where 70% of the global HIV-1 infections occur and where vaccination is the only rational strategy for an affordable prevention against HIV-1 infection (McCutchan, 2000; Peeters and Sharp, 2000; UNAIDS, 2000). In particular, the latest data produced by the UNAIDS HIV Network indicate that the A clade accounts for 25% of all HIV-1 isolates identified world wide and is predominant in many developing countries, including Uganda where a prevalent A and D 'bi-clade' HIV-1 epidemic has been described (Albert et al., 1992; Bruce et al., 1994; Buonaguro et al., 1995; Brennan et al., 1997; Rayfield et al., 1998).

Candidate therapeutic vaccines, based on cross-clade conserved regulatory HIV-1 proteins (namely native or detoxified Tat protein), have been recently tested in primates and humans with significant induction of humoral and cellular immune response (Zagury et al., 1998; Cafaro et al., 1999; Gringeri et al., 1999). Although the Tat-based strategy may be effective for the control of HIV-1 disease progression, its possible role as a preventive vaccine is still controversial (Gallo, 1999; Pauza et al., 2000).

Therefore, in the perspective of a global HIV-1 preventive vaccine program, candidate vaccines based on non-B-clade derived structural antigens should be developed and eventually used in a cocktail formulation with B-clade antigens, in order to have better chances of efficacy.

The results in the present study indicate that the HIV-VLP_As show a strong in vivo immunogenicity in Balb/c mice and induce both humoral and cellular immunity, in the absence of adjuvants. In particular, both HIV-1-specific CTLs and cross-clade neutralizing antibodies have been detected in immunized animals, indicating that such approach represents a promising vaccine strategy.

2. Materials and methods

2.1. Production of recombinant baculovirus and HIV-VLP_As.

Recombinant Baculoviruses expressing both the $gp120_{UG}$-TM fusion gene and the Pr55gag cDNA were produced as previously described (Buonaguro et al., 2001). Briefly, the HIV-1 coding sequences have been transferred in a single-step, by site-specific transposition, from the pFastBac Dual transfer vector (Gibco-BRL) to the Baculovirus DNA. This is obtained in a DH10Bac™ *E. coli* bacterial strain (Gibco-BRL) modified to propagate both a baculovirus shuttle vector (bacmid), which contains mini-attTn7 attachment sites, and the pMON7124 helper plasmid, which provides in trans the Tn7 transposition functions. The recombinant Baculovirus DNA was used to transfect SF9 insect cells and supernatants were collected at 48–72 h post-transfection; different Multiplicities of Infections (MOI) were then used to infect High Five™ cells and scale up the production of VLPs. Particles were purified through a continuous 10–60% sucrose gradient (Buonaguro et al., 2001).

2.2. Immunization experiments

Four groups of male Balb/c mice, each group consisting of three animals 6–8 weeks of age, were injected intraperitoneally (i.p.) with increasing doses of purified VLPs (5, 10 or 20 μg) resuspended in 200 μl of PBS. The immunization schedule was based on a 4-dose regimen, where the booster inoculations were administered at weeks 3, 7 and 9 after the primary injection; serum samples were collected at each inoculation by blood drawing from the retro-orbital vein. All animals were sacrificed at week 11 by cervical dislocation, with collection of serum samples and removal of spleens for immunological assays. Alternatively, for the evaluation of induction of cellular immunity, mice were injected i.p. with a single dose of 5 or 20 μg of VLPs, resuspended in 200 μl of PBS, and spleens were removed 1 week post-injection. All the immunization protocols were performed in two independent experiments without addition of adjuvants.

2.3. Serological assays

The presence and the titer of specific anti-Gag and anti-Env antibodies was evaluated in serum samples obtained from immunized mice by an enzyme-linked immunosorbent assay (ELISA) as well as by Western Blot analysis. To this end

96-well MICROTEST assay plates (Becton Dickinson) were coated either with 0.5 μg of the gp120-Env V3 loop peptide (37 aa), representing the consensus of the Ugandan A-clade HIV-1 strains, or with 0.5 μg of 20 overlapping peptides (20 aa each) spanning the entire p24-Gag protein. Plates were incubated with dilutions of mouse sera ranging from 1:800 to 1:204800; positive reactions were visualized with 0.075% 4-chloro-1-naphthol in 0.056% hydrogen peroxide and stopped with 2N sulfuric acid. Absorbance was determined at 492 nm and reactions were considered positive when exceeding by a factor of 5 the mean absorbance of equal dilutions of sera collected from control animals. The anti-Gag and anti-Env antibody levels were evaluated as the geometric mean titer of the last positive dilution of sera from the animals of each set, in the two independent experiments.

2.4. HIV-1 neutralization assay

The neutralization assay was performed using as target the U87 human glioma cell lines transfected with the CXCR4 or CCR5 chemokine receptors (Cecilia et al., 1998). The homologous 94UG018 field isolate, used for the assays, is a primary isolate identified in an Ugandan asymptomatic recently sero-converted individual by co-culture with PBMCs, pooled from two different HIV-1-seronegative donors, and frozen down with no additional passages on lymphocyte cell lines (Buonaguro et al., 1995). The 94UG018 field isolate replicates only on CCR5-expressing U87 cells and, in these cells, it has been titrated for the ex vivo neutralization assays. No signs of replication were detectable in CD4 or CD4/CXCR4-expressing U87 cells. U87 target cells were seeded in 96-well plates, at a density of 1×10^4 cells/well. On the following day, 10 $TCID_{50}$ of both 94UG018 and IIIB viruses were mixed with serial dilutions of mouse serum (1:20–1:80) and incubated for 1 h at 37 °C. The serum was derived from the mouse group immunized with 20 μg of HIV-VLP_As, at the end of the complete immunization schedule. The virus–serum mixture was added to well-adherent CCR5-expressing U87 cells (for the 94UG018 virus) or CXCR4-expressing U87 cells (for the IIIB virus), in triplicate, and incubated overnight. After three washings with phosphate-buffered saline, 200 μl of complete medium was added per well and cell cultures were kept for 7 days. The levels of HIV-1 p24 antigen in the supernatants was determined at day 4 and 7 post-infection (p.i.), by a commercially available kit (Nen DuPont); percent of neutralization was calculated relatively to a dilution-matched non-treated control. The test was scored positive when the serum dilution exhibited a $> 50\%$ neutralization effect.

2.5. Env-specific T cell proliferative responses

In vitro proliferative responses were determined as previously described (Boehncke et al., 1993). Single-cell suspensions of spleens were obtained from immunized and control mice, and cultured in RPMI medium supplemented with 10% FCS. Then, 5×10^5 cells were stimulated in 96-well plates in triplicate with different doses (1–5 μg) of recombinant gp120 ($rgp120_{MN}$) (Intracell) for 5 days; cells were then pulsed for 12 h with 1 μCi/well of ^{3}H-thymidine. The proliferation index in cpm (counts per minute) was calculated as the average ($\pm$ s.d.) from the triplicate results of all three animals per set point in the two independent experiments. The stimulation index (S.I.) was calculated by dividing the proliferation index of the cultures stimulated with rgp120 by the proliferation index of control cultures.

2.6. Cytotoxic T lymphocyte assay

Single-cell suspensions of spleens were obtained from immunized and control mice and co-cultured in presence of IL-2 (5 IU/ml) for 7–14 days with irradiated Env- or Gag-expressing syngeneic splenocytes (stimulators), in order to propagate specific effector T cells. In particular, stimulator cells were induced for 24 h with 5 μg/ml of phytohemagglutinin (PHA, Sigma), infected with 1 PFU/10^3 cells of Env- or Gag-recombinant vaccinia virus and, after additional

16–24 h, γ-irradiated with 2,000 rads. For cytolytic assays, target syngeneic H-2^d P815 cells were infected with 1 PFU/10^3 cells of either Gag- or Env-vaccinia virus, 16–24 h prior to the start of the assay. Both Gag- and Env-vaccinia virus were obtained from the NIBSC Centralised Facility for AIDS Reagents; the *gag* sequence was derived from the B clade HIV-1_{BH10} isolate (Nixon et al., 1988) and the *env* sequence from an Ugandan A-clade HIV-1 isolate (Gao et al., 1994). Infected target cells were pulsed for 1 h at 37 °C with ^{51}Cr (Amersham Pharmacia Biotech) (30 μCi/10^6 cells) and washed three times with culture medium before the addition of effector cells. Specific lysis was measured in a triplicate assay performed with 5×10^3 target cells mixed with different ratios of effector cells (25:1 to 3.125:1) (E:T) in a total volume of 200 μl. After a 5-h incubation at 37 °C, 100 μl culture supernatant was collected for counting the amount of ^{51}Cr release. Positive and negative controls, in absence of effector cells, were treated with or without 1N HCl, respectively. The percentage of specific lysis was calculated as follows: 100 × (specific release − spontaneous release)/(total release − spontaneous release).

3. Results

3.1. In vivo humoral immune response induced by HIV-VLP_As

Induction of a humoral immune response by HIV-VLP_As was evaluated in a Balb/c mouse model. Twelve animals, divided in four groups, were used for a four-dose immunization schedule with HIV-VLP_As produced in High-Five™ insect cells (Gibco BRL) and purified through a 10–60% continuous sucrose gradient (Buonaguro et al., 2001). In two independent experiments, three animal groups were treated with an i.p. injection of 5, 10 or 20 μg purified VLPs resuspended in phosphate-buffered saline (PBS), respectively; the control group was i.p. injected with PBS alone. At weeks 3, 7 and 9 each group was boosted with the same amount of antigen as for the primary injection and, concomitantly, blood samples were drawn from the retro-orbital vein. With the exception of one animal from the 10-μg group in one of the two experiments, which died for unrelated reasons after the first booster dose, all other animals did not show signs of toxicity and remained healthy up to the end of the vaccination protocol. In order to evaluate specific antibody titers induced by HIV-VLP_As, ELISA tests were performed on microwell plates coated either with a single V3 peptide, representing the Env consensus of Ugandan A-clade HIV-1 strains, or a series of overlapping peptides spanning the whole p24-Gag protein, derived from a B-clade HIV-1 strain. Serum Ab titration, with a dilution range from 1:800 to 1:204800, showed that the multi-dose regimen immunization protocol was highly effective, given that all three VLP doses elicited a final antibody mean titer of 1:102400. In particular, the 10- and 20-μg dose elicited equivalent antibody titers against both antigens, with levels of 1:12800 already 3 weeks after the primary injection (Fig. 1A and Fig. 1B). Reactions were considered positive when the absorbance at 492 nm exceeded by a factor of 5 the average absorbance of equal dilutions of sera collected from control animals.

3.2. Neutralization activity against autologous and heterologous virus strains

The neutralization activity of sera from mice immunized with 20 μg of HIV-VLP_As, by the multi-dose regimen, was verified against the autologous primary field isolate (94UG018) as well as a heterologous virus strain, the T-cell line adapted (TCLA)–HIV-1_{IIIB}, using as target the CR-expressing U87 human glioma cell line. Replication of the 94UG018 isolate in CCR5-expressing U87 cells was significantly inhibited by the immune sera during the 7 day p.i. incubation, up to a final dilution of 1:40 (Fig. 2A). In particular, the neutralization curves indicate that such sera dilution had, on average, an 80% and a 70% neutralization capacity, on day 4 and 7 p.i., respectively. On the contrary, a 45% neutralization was transiently observed on day 4

p.i., with a serum dilution of 1:80, but was no longer detectable on day 7 p.i. (Fig. 2B). The highest neutralizing serum dilution (1:40) was also tested against the heterologous TCLA HIV-1_{IIIB} strain, using as target the CXCR4-expressing U87 cells. Virus replication was significantly reduced and, in particular, an average 60% neutralization was observed on day 4 and 7 p.i. (Fig. 2C and Fig. 2D). In both the control and immunized groups, the standard error of the means was always lower than 5% of the mean value.

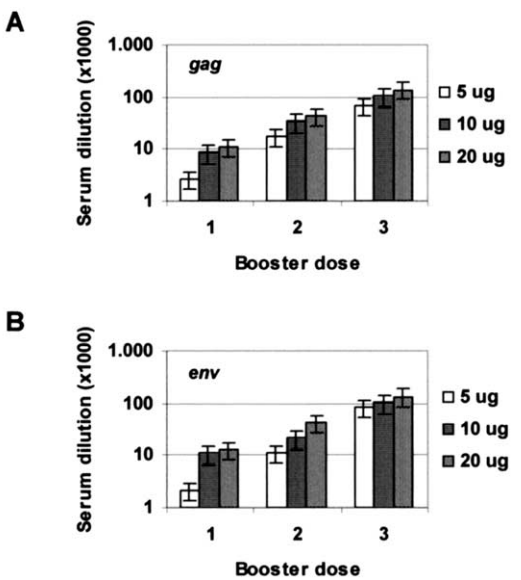

Fig. 1. Levels of anti-Gag (A) and anti-Env (B) humoral response induced in VLP-immunized mice. The histogram represents the geometric mean titer ($\pm$ s.d.) of anti-HIV-1 specific antibodies induced in each Balb/c mouse group by i.p. inoculation of HIV-VLP_As (5, 10 or 20 μg), in two independent experiments. At 3, 7 and 9 weeks following the primary immunization, all three animal groups were bled and received a booster with same antigen doses of the corresponding group. Serum samples were assayed for specific antibodies by ELISA, performed in triplicate on 96-well plates coated with 0.5 μg of overlapping peptides spanning the p24-Gag protein (A) or gp120-Env V3 consensus peptide (B). For both, the geometric mean endpoint titer from each group of animals is shown as the reciprocal of the last dilution giving a fivefold OD_{492} of the pre-immune sera.

3.3. In vivo T cell activation induced by HIV-VLP_As

To assess the stimulation in vivo of Env-specific T helper cells, spleens were collected from Balb/c mice immunized with HIV-VLP_As, either by a single inoculation or by a multi-dose regimen, in absence of adjuvants. In both cases, single-cell suspensions of splenocytes were cultured in complete RPMI and stimulated for 5 days with rgp120 (HIV-1_{MN} strain). In order to identify the optimal experimental conditions, splenocytes from controls and mice immunized with 20 μg of VLPs were treated with increasing amounts of $rgp120_{MN}$. The proliferation of T helper cells from immunized animals showed a dose–response pattern, with a plateau at 2–4 μg of rgp120 (S.I. mean value = 5.3), while T cells from controls showed a persistent baseline proliferation (S.I. mean value = 1) (Fig. 3A). The 2-μg dose of rgp120 was then used to evaluate the degree of T cell proliferation in the three groups of immunized animals. The animal group injected with the 5-μg VLP dose did not exhibit a sufficient in vivo priming and T cells showed only a baseline response to $rgp120_{MN}$ (S.I. mean value = 1.1); on the contrary, an induction was observed in the animal groups injected with the 10- and 20-μg VLP doses and a specific T cell proliferation was detected with an S.I. of 2.4 and 5.3, respectively (Fig. 3B). These data suggest that the S.I. values and the VLP doses are characterized by a highly significant linear correlation ($r = 0.94$) (Fig. 3B). In each group for each experiment, the s.d. was always lower than 10% of the mean value; moreover, the standard error of the means was always lower than 5% of the mean value. For both groups (10- and 20-μg dose group), the fold of stimulation of Env-specific T-helper proliferation, over T-helper proliferation of non-immunized control animals, was statistically significant (P value < 0.001).

No significant differences were observed between the two immunization schedules characterized by a single inoculation or by a multi-dose regimen. These results suggest that the $gp120_{UG}$-TM molecules exposed on the HIV-VLP_As surface are able to induce an efficient in vivo priming of T helper cells.

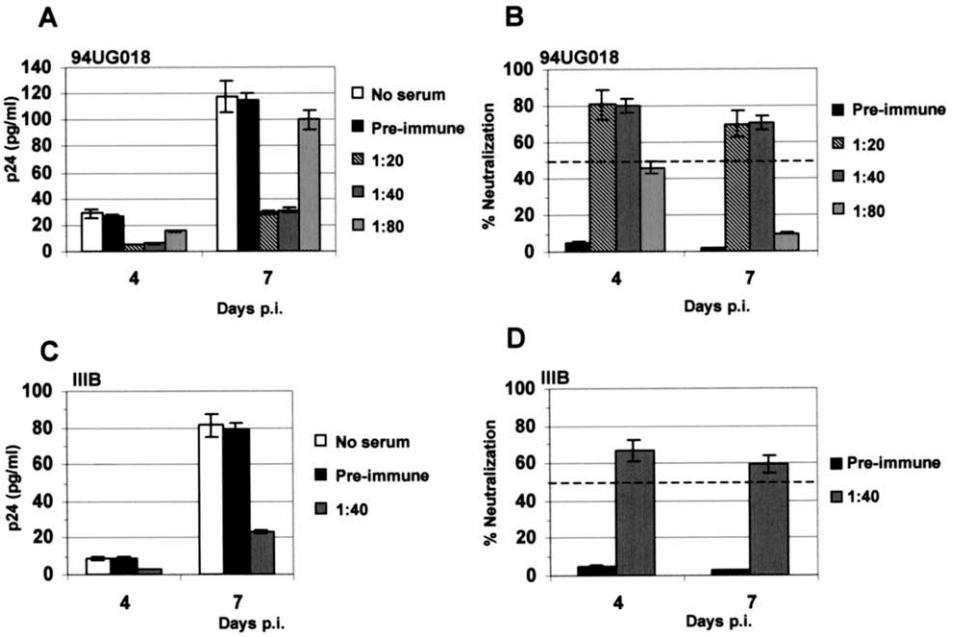

Fig. 2. Homologous and heterologous neutralization efficiency of sera from VLP-immunized mice. The neutralization analysis was performed against the homologous HIV-1 94UG018 isolate, in the CCR5-expressing U87 cell line, and the heterologous HIV-1_{IIIB} isolate, in the CXCR4-expressing U87 cell line. Ten $TCID_{50}$ of each isolate were pretreated with serial dilutions of the immune sera from the mouse group immunized with 20 μg of HIV-VLP_As, by the multi-dose regimen, at the end of the complete immunization schedule. The kinetics of infection of the HIV-1 94UG018 and the HIV-1_{IIIB} isolates, in the specific CR-expressing target U87 cell line, is shown in panel A and C, respectively. The positive control in both panels is represented by the viral growth of non-treated virus (□ No serum). The neutralization of 94UG018 (B) and HIV-1_{IIIB} (D) isolates, at different serum dilutions, is shown as percentage of the virus replication in control samples. The dashed line indicates the 50% neutralization, which has been considered the lowest limit for a positive test. Each serum dilution has been evaluated in triplicate and the reported results are representative of two independent experiments. The standard error of the means was lower than 10% of the mean value.

3.4. Cytotoxic T lymphocyte response to HIV-1 epitopes

To assess the induction of Env- and Gag-specific CTL, Balb/c mice were immunized with HIV-VLP_As either by a single inoculation or by a multi-dose regimen, in the absence of adjuvants, in two independent experiments. Splenocytes (effectors) were purified from immunized animals and in vitro re-stimulated for 2 weeks with γ-irradiated splenocytes infected with either Env- or Gag-vaccinia recombinants. In particular, the *env* sequence was derived from an Ugandan A-clade HIV-1 isolate (Gao et al., 1994) and the *gag* sequence from the B clade HIV-1_{BH10} isolate (Nixon et al., 1988). The cytotoxic activity of effectors was then evaluated in a ^{51}Cr-release assay against syngeneic (H-2^d) P815 cells (targets) infected with the same Env- or Gag-vaccinia recombinants, using an Effector:Target (E:T) ratio ranging from 25:1 to 3.125:1. A 26–30% CTL response was detected at the E:T ratio of 25:1 in each paired experiment performed with splenocytes from animals immunized with 20 μg VLPs. These experiments were performed using for both the in vitro restimulation and the lysis assay either the HIV-1 env or the gag epitopes, independently. The correlation between the percentage of observed lysis and the E:T ratio was approximately linear within the analyzed range (Fig. 4A and Fig. 4B). Less than 15% CTL response was observed at the 25:1 E:T ratio in experiments performed with splenocytes from animals immunized with 5 μg VLPs (11% for env and 13% for gag). On the

contrary, a $< 5\%$ lysis was observed when the effectors were re-stimulated in vitro with Env and mixed with Gag-expressing targets (and vice versa), indicating that the reaction was specifically driven in vitro by antigen-re-stimulated CTL clones. As expected, no cytotoxic activity was detected against parental P815 (Fig. 4A and Fig. 4B). Each point of these evaluations was performed in triplicate and the s.d. was always less than 10% of the mean value. No significant differences have been observed between the two immunization schedules characterized by a single inoculation or by a multi-dose regimen.

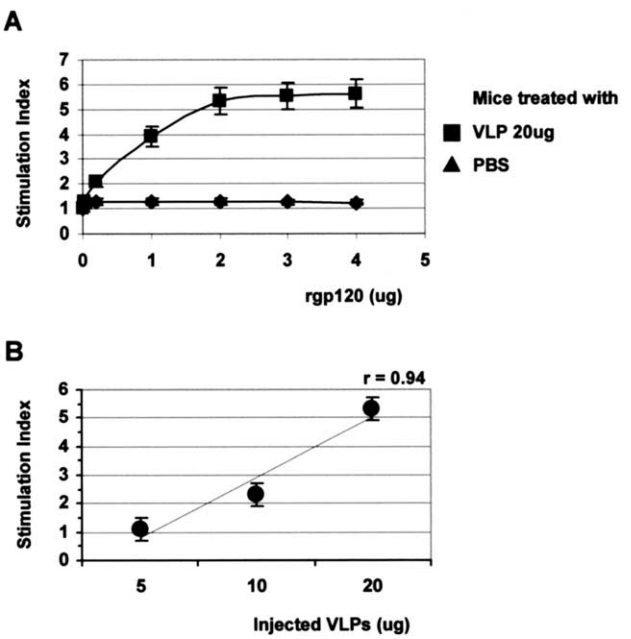

Fig. 3. T helper cell activation in VLP-immunized mice. Cellular T helper response was evaluated in each mice group inoculated with HIV-VLP$_A$s (5, 10 or 20 μg). (A) The panel shows the Stimulation Index (S.I.) pattern of splenocytes from the PBS control and the 20-μg immunized group, stimulated with increasing doses of rgp120$_{MN}$ (Intracell). (B) The panel shows the S.I. pattern of splenocytes purified from the three immunized groups and in vitro stimulated with 2 μg of rgp120$_{MN}$. S.I. represents the fold increase of thymidine incorporation in cells from immunized animals compared to cells from control animals. The S.I. values and the the VLP doses show a highly significant linear relationship ($r = 0.942$). In both panels, the mean value obtained for each animal group in two independent experiments is shown; the standard error of the means was always lower than 10% of the mean value, with a P value < 0.001.

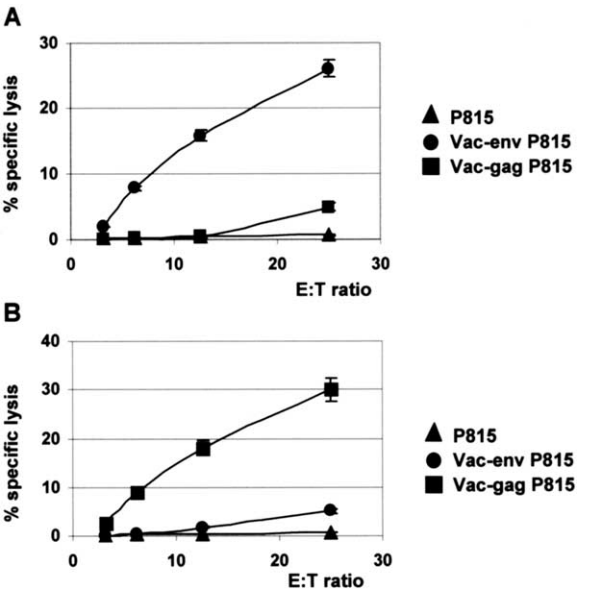

Fig. 4. T cytotoxic cell activation (CTL analysis) in VLP-immunized mice. The CTL response was evaluated in splenocytes from immunized mice, inoculated with a single dose of 20 μg of HIV-VLP$_A$s. Splenocyte cultures were prepared 2 weeks after inoculation and stimulated in vitro with γ-irradiated splenocytes infected with either Env-(A) or Gag-(B) expressing vaccinia recombinants. Effector cells were then assayed for lysis of P815 target cells presenting either HIV-1 gp160$_A$ or Gag$_B$ epitopes. Parental P815 cells were used as negative control in both experiments. Specific cell lysis was measured as ^{51}Cr release and expressed as the percentage of total lysis from acid-disrupted target cells. Data shown in the figure are the mean values of triplicate cultures from each of the two independent experiments. The standard error of the means was always lower than 5% of the mean value.

4. Discussion

The main objective of this study was to characterize the immunogenicity of a candidate HIV-1 preventive/therapeutic vaccine recently developed in our laboratory, based on VLPs expressing a gp120 glycoprotein derived from a HIV-1 isolate of the A clade (Buonaguro et al., 1998a, 2001). This clade represents, in fact, the 25% of all HIV-1 isolates identified world wide and is predominant in many developing countries, including Uganda where a prevalent A and D 'bi-clade' HIV-1 epidemic has been described (Albert et al., 1992; Bruce et al., 1994; Buonaguro et al., 1995; Brennan et al., 1997; Rayfield et al., 1998).

The immunogenicity of the HIV-VLP$_A$s was evaluated in Balb/c mice and the induction of both humoral and cellular immune response was observed in absence of adjuvants. An immunization protocol based on a four-dose regimen, performed in parallel in three animal groups with three different doses of HIV-VLP$_A$s (5, 10 and 20 μg, respectively), elicited increasing levels of anti-V3 and anti-p24 antibodies, up to a final antibody titer of 1:102400.

Immune sera were tested for neutralization activity against both the homologous and a heterologous strain, using the CR-expressing U87 human glioma cell lines as target. The use of U87 cell line as target cells in neutralization assays, as previously described for the GHOST osteosarcoma cell line expressing the chemokine receptors, significantly reduced the variability normally observed in PBMC-based assays, eliminating discrepancies related to donor susceptibility and co-receptor expression (Cecilia et al., 1998). In particular, CCR5-expressing cells have been used for the neutralization assays with the 94UG018 primary isolate, whereas the CXCR4-expressing cells have been used for the neutralization assays with the T-cell adapted HIV_{IIIB} isolate. Sera from animals immunized with 20 μg of HIV-VLP$_A$s showed a neutralization activity against both the 94UG018 homologous field isolate ($>70\%$ neutralization) and the heterologous, T-cell adapted, HIV-1_{IIIB} isolate ($>60\%$ neutralization), at a final dilution of 1:40. The higher neutralization efficacy of the sera from immunized mice against the homologous primary isolate is extremely significant, given the reported general poor neutralization sensitivity of primary isolates (Montefiori et al., 1998; Trkola et al., 1998). These data indicate that the chimeric gp120-TM, anchored on the surface of VLPs through the EBV heterologous trans-membrane moiety, could acquire a peculiar three-dimensional conformation which induces antibodies with equivalent or higher neutralizing activity against epitopes presented on primary isolates than epitopes unshielded on the TCLA strain. The nature of such epitopes needs to be further verified on additional homologous as well as heterologous HIV-1 primary isolates from early and late stage of infections. In fact, either a cross-clade neutralization against different envelope molecules or a cross-neutralization against additional epitopes, unshielded in different stages of infections, would be of extreme relevance for properties and applications of the VPL vaccine approach.

A cellular T helper response was observed in Balb/c mice treated either with a single inoculation or with a multi-dose regimen. No significant differences were observed between the results obtained with the two protocols, indicating that multiple injections of VLPs and addition of adjuvants are unnecessary to obtain an efficient T helper stimulation. The maximum degree of in vivo T cell stimulation was obtained with the 20-μg dose of HIV-VLP$_A$s, which, in our experimental conditions, elicited also the highest humoral immune response. It is noteworthy to mention that the ex vivo helper T cell proliferative response was observed using, as stimulating antigen, a rgp120 derived from a B-clade HIV-1_{MN} strain, presenting a 18.6% divergence with the entire $gp120_{UG}$ (Buonaguro et al., 1998b).

Furthermore, a single inoculation of 20 μg of HIV-VLP$_A$s was able to induce in vivo $CD8^+$ CTLs, and a specific cytolytic activity was observed against target cells expressing either Env or Gag molecules derived from HIV-1 isolates of the A and B clades, respectively. These observations indicate that VLPs, due to their particulate structure, represent powerful inducers of CTL response since they can be processed by antigen-presenting cells and presented to the immune system in a MHC class I context. The results are concordant with recent reports on the induction of strong and long-lasting cellular immune responses following HIV-VLP immunization in mice as well Rhesus macaques (McGettigan et al., 2001; Paliard et al., 2000).

All the results relative to the humoral and cellular immune responses were obtained in two independent immunization experiments, where the s.d. was comprised between 5 and 10% of the mean values and the standard error of the means was $<5\%$ of the mean value, indicating a significant consistency of the independent observations.

The data described here indicate that Pr55gag-based VLP is one of the promising HIV-1 vaccine

candidates, which should be further verified for its immune-protective efficacy in non-human primates. It is suitable for an ideal presentation of multiple linear or conformational epitopes with an effective induction of both arms of the immune response, in absence of adjuvants (Griffiths et al., 1993; Wagner et al., 1996; Deml et al., 1997; Tobin et al., 1997; Montefiori et al., 2001). As further support for its scientific validity, the VLP concept is currently under investigation as a potential vaccine for other human and non-human viruses, such as hepatitis, papilloma, pseudorabies and equine herpes viruses (Kirnbauer et al., 1992; Garnier et al., 1995; Osterrieder et al., 1995; Li et al., 1997).

VLPs have been already evaluated in two phase I clinical trials as a potential HIV-1 vaccine strategy. In particular, the immune therapeutic potential of HIV-1 VLPs, expressing only the p17/p24 core proteins, has been assessed in HIV-1-seropositive individuals, without significant improvements in clinical and virological parameters of the HIV-1 infection (Klein et al., 1996; Kelleher et al., 1998). In both studies, however, the VLPs were administered in patients with high viremia and, therefore, with an overload of viral antigens, which could have impaired the therapeutic effects. Current opinion, in fact, is that the administration of therapeutic vaccines should follow a short efficacious antiretroviral treatment, achieving a reduction of the plasma viral load (Rosenwirth et al., 1999).

Furthermore, an analogous HIV-VLP vaccine, expressing a gp120 derived from a B clade isolate, has recently been tested in Rhesus macaques, where, despite a significant induction of both CTL and NA, no protection from a challenge with homologous chimeric simian/human immunodeficiency virus (SHIV) was afforded (Wagner et al., 1998). Several indications, however, suggest that such failures might be ascribed not only to vaccine strategies but also to immunization protocols. Experimental evidences, in fact, indicate that only some vaccination schedules, with time-sequential injection of specific antigen preparations, show very promising results (Almond and Heeney, 1998). In this respect, the evaluation of the immune-protective capacity of our HIV-VLP_As, in the context of multiple combination strategies, will represent one of the main objectives of our future studies in non-human primates.

The HIV-1 vaccine evaluation studies are, in general, hampered by the lack of an effective non-human primate model susceptible to HIV-1 productive infection. The use of macaques, challenged with chimeric SHIVs, represents an inevitable surrogate for assessing HIV-1 vaccine strategies. Engineered SHIVs can have biological properties different from HIV-1 in humans and, consequently, the results of protection from challenge could not be predictive of vaccine efficacy in humans (Letvin, 1998). Furthermore, the bio-engineered SHIVs are limited in number (Thakallapally et al. 1999) and the prevalence of B-based chimeras has so far hampered the possibility of detecting immune-protective responses against HIV-1 isolates predominant in non-B areas.

Thus, given the restrictions imposed by the available animal models, a significant end-point is represented by the induction of a strong humoral and cellular immune response, which should ensure the containment of virus replication as well as the progression of infection. Such levels of immune protection in primates may be sufficient to expect a reduction of viral spread in humans and, ultimately, progressive containment of the HIV-1 epidemic (Letvin, 1998; Shen and Siliciano, 2000). This would be particularly relevant for countries with high transmission rate and low possibility of a continuous treatment with expensive and life-lasting anti-retroviral drugs.

Finally, since almost all candidate HIV-1 vaccines, advanced into clinical trials, are based on antigens derived from HIV-1 B clade, the development of vaccines based either on antigens derived from non-B HIV-1 strains or on a cocktail of epitopes derived from different HIV-1 clades, is a prerequisite in the perspective of a global HIV-1 preventive vaccine program. The HIV-VLP_As comply with such necessity and their employment, along with more conserved antigens, might be helpful for protocols specifically targeted to sub-Saharan Countries, in particular Uganda, for a therapeutic as well as preventive vaccine approach.

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Recombinant vaccinia virus, expressing gp160 of HIV-1 A clade (EVA 289.1–4) and Pr55gag of HIV-1 B clade (ARP 253), gp120 V3 peptide (EVA7017.1) and overlapping peptides spanning the entire p24-Gag protein (ARP788.1–22) were obtained through the NIBSC Centralised Facility for AIDS Reagents supported by EU Programme EVA (contract BMH4 97/2515) and the UK Medical Research Council. The original donor of the reagents were Dr. M. Esteban (EVA 289.1–4) and Dr. D. Nixon (ARP 253). CXCR4- and CCR5-transfected U87 human glioma cell lines were kindly provided by Dr. L. Lopalco, San Raffaele Scientific Institute, Milano, Italy. We thank Mr. John McKnight for revising the English style of the manuscript. This study was supported by grants from the Ministero Italiano della Sanità (Ricerca Corrente and Progetto Finalizzato AIDS 1999) and the ICSC-World Lab, Lausanne, Switzerland (Project MCD-2/7).

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