In vivo induction of cellular and humoral immune responses by hybrid DNA vectors encoding simian/human immunodeficiency virus/hepatitis B surface antigen virus particles in BALB/c and HLA-A2-transgenic mice

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Abstract

To improve the immunogenicity of epitopes derived from Gag proteins of simian immunodeficiency virus (SIV) and from the envelope (Env) protein of human immunodeficiency virus type 1 (HIV-1), we have designed hybrid DNA vaccines by inserting sequences encoding antigenic domains of SIV and HIV-1 into the hepatitis B virus envelope gene. This gene encodes the hepatitis B surface antigen (HBsAg) capable of spontaneous assembly into virus-like particles that were used here as carrier. Injections of hybrid vectors encoding B-cell epitopes from the gp41 and the gp120 envelope proteins of HIV-1 induced specific humoral responses in BALB/c mice. Furthermore, high frequencies of IFN-γ-secreting $CD8^+$ T cells specific for various antigenic determinants of SIV-Gag were observed after intramuscular injections of hybrid DNA vectors in BALB/c mice. Genetic immunization of HLA-A2.1-transgenic mice with HIV-Env/HBsAg-encoding DNA generated a strong CTL response and IFN-γ-secreting $CD8^+$ T lymphocytes specific for HIV-1 envelope-derived peptide. H-2^d-restricted HBs-specific T-cell responses dominated over SIV-Gag responses in BALB/c mice whereas HLA-A2-restricted HIV-Env response was enhanced after fusion with HBsAg. These data demonstrate that different B and T-cell epitopes of vaccine-relevant viral antigens can be expressed in vivo as fusion proteins with HBsAg but that the optimal immunogenicity may differ strikingly between individual epitopes.

Keywords: Hybrid DNA vaccine

Abbreviations: APC, antigen presenting cells; CMV, cytomegalovirus; Env , Envelope ; HBsAg , hepatitis B surface antigen ; hybrid DNA, hybrid SHIV/HBsAg particles-expressing DNA; HIV-1, human immunodeficiency virus type 1; i.m., intramuscular; MOI, multiplicity of infection; SFC, spot forming cells; SHIV, simian/human immunodeficiency virus; SIV, simian immunodeficiency virus; VLP, virus-like particle

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Introduction

The development of an effective vaccine is an urgent goal to control the human immunodeficiency virus (HIV) epidemic. In infected individuals, cellular immune response has been shown to be important in the early control of viral replication (see McMichael and Rowland-Jones (2001) for a review). In simian

immunodeficiency virus (SIV) infection of macaques, the suppression of $CD8^+$ T cells is followed by an increase in viremia and an accelerated disease progression (Schmitz et al., 1999). Strong HIV-specific CTL responses have been demonstrated in cohorts of infected individuals with slow development of the symptoms of the disease (long-term non-progressors) (Rowland-Jones et al., 1997). An effective HIV-1 vaccine should induce strong and sustained cellular responses, for example against conserved HIV-1 epitopes recognized by cross-clade CTL responses, such as Gag epitopes (Durali et al., 1998; McAdam et al., 1998). However, during AIDS progression, the number of HIV-specific tetramer-positive $CD8^+$ T cells is maintained but T-cell response based on IFN-γ production is progressively impaired (Kostense et al., 2002) suggesting that CTL by themselves are not sufficient to efficiently control HIV replication.

For controlling HIV-1 infection, $CD4^+$ helper T cells have a critical regulatory role as their activation is required for the development of memory $CD8^+$ T-cell and humoral responses. The proliferative HIV-specific $CD4^+$ response has been associated with the control of viral load and correlated with a slow disease progression (Rosenberg et al., 1997). The humoral response is also required to prevent viral entry into target cells. Experiments in chimpanzees involving passive transfers have shown that monoclonal antibodies to HIV-1 envelope provide protection against challenge with homologous virus (Emini et al., 1992). DNA vaccines have been shown to elicit both HIV-specific CTL and humoral responses in animal models (Gurunathan et al., 2000; Garber et al., 2004). The safety of DNA vaccine trials has already been proved in humans infected or not by HIV (Calarota and Weiner, 2004) but their potency in controlling viral replication has still to be improved.

DNA immunization enhances mainly cytotoxic responses but the mechanism underlying the induction of immune response after intramuscular (i.m.) DNA vaccination is still unclear. Dendritic cells can be directly transfected by the injected DNA and thus present endogenously synthesized antigens via the classical cytosolic MHC I pathway and prime the $CD8^+$ T-cell response (Condon et al., 1996; Akbari et al., 1999). Alternatively, directly transfected muscle cells may express the protein antigen, and transfer it to APC to induce the T-cell response by cross priming (Ulmer et al., 1996; Tighe et al., 1998; Bahjat and Schoenberger, 2004). DNA vaccines can also encode fusion proteins such as antigenic protein fused to a carrier that may help the targeting to APC and the loading through MHC I and II pathways. For example, the use of virus-like particles (VLPs) as carrier is a potent strategy to induce cross presentation of antigens and CTL activation (Moron et al., 2002). This strategy also activates high levels of T-cell help, the key to induction and maintenance of effective immunity (Stevenson et al., 2004; Wherry and Ahmed, 2004).

In the present study, we constructed hybrid DNA vectors encoding antigenic domains of SIV or HIV-1 fused to HBsAg as a carrier. HBsAg can incorporate up to 395 AA, still forming particles (Michel et al., 1988; Bisht et al., 2002). The use of HBsAg encoding DNA as a genetic carrier takes advantage of DNA immunization and should allow the long-lasting production and secretion of pseudo-particles presenting SIV or HIV antigenic sequences that can be processed in the context of class I and II MHC pathways (Bohm et al., 1995; Schirmbeck et al., 1995; Malanchere-Bres et al., 2001). These constructs were designed for further use in Rhesus monkeys. Three SIV-Gag/HBsAg-encoding DNA were first injected in BALB/c mice in order to test their immunogenicity. Three HIV-Env/HBsAg hybrid DNA vectors were also designed. One of them encoding a HLA-A2-restricted HIV-Env T-cell epitope was tested in HLA-A2-transgenic mice, the two others encoding HIV-Env B-cell epitopes were injected in BALB/c mice to study the humoral response. We show that SIV-Gag-, HIV-Env-specific CTL as well as HBV-specific CTL were induced, and high frequencies of specific IFN-γ-secreting $CD8^+$ T cells were detected. Furthermore, immunizations with hybrid vectors encoding HIV-Env B-cell epitopes induced a humoral response specific for gp41 and gp120 in mice.

Materials and methods

Generation of hybrid vectors

We used pCMV-B10 construct (Firat et al., 1999) which encodes the small (S) and part of the middle (preS2 + S) envelope proteins of the hepatitis B virus envelope under the control of the cytomegalovirus (CMV) immediate early gene promoter (Fig. 1). The central part of the sequence coding for the preS2 domain

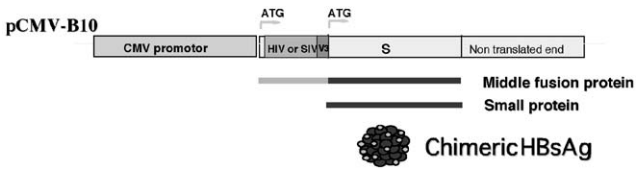


Fig. 1. Hybrid DNA vector used as vaccine in mice and proteins expressed by the vector. pCMV.B10 recombinant expression vector is derived from pCMV-S2.S vector. The central part of the pre-S2 coding sequence was replaced by in frame HIV or SIV sequences encoding antigenic domains. Hybrid DNA codes for the small HBV envelope protein and for hybrid middle proteins that self-assemble into chimeric HBsAg particles.

was replaced by a polylinker and a coding sequence derived from the HIV-1 V3 loop as a tag. The domains selected for insertion in the pCMV.B10 have been shown to be immunogenic in SIV-infected Rhesus monkeys (Korber et al., 2004). DNA fragments encoding amino-acid residues 165–195 (Gag I), 246–282 (Gag II) and 165–282 (Gag III) of the central region of the SIV mac 239 Gag protein are recognized by CTL in SIV infected monkeys (Allen et al., 1998; Korber et al., 2004). Domains belonging to the HIV 89.6 Env protein were also selected: the residues 104–130 (Env I) from the external glycoprotein gp120 contains a highly conserved CTL epitope between SIV, HIV-1 and HIV-2 isolates (Voss and Letvin, 1996), and the residues 548–561 (Env III) belonging to the transmembrane gp41 protein induce CTL in the infected monkey (Voss and Letvin, 1996). Two domains coding for B-cell epitopes: the residues 300–332 (Env II) in the third hypervariable region of the gp120 envelope glycoprotein, and the residues 655–670 (Env IV) in the gp41 envelope protein known to induce neutralizing antibody response (Muster et al., 1993), were selected as B-cell epitopes. Hybrid DNA vectors encoding SHIV 89.6P antigenic domains fused to HBsAg were constructed by PCR amplification or using partially complementary oligonucleotides. We used pCI vectors carrying the complete *gag* gene of SIV or the *env* gene of HIV-1 (Habel et al., 2000) as a template for PCR amplification. DNA fragments corresponding to nucleotides 493–585, 736–846 and 493–846 of the SIVmac239 *gag* gene, and 310–390 and 898–996 of the HIV-1 *env* gene were amplified by PCR and inserted into a Bluescript plasmid (Stratagene, Saint Quentin en Yvelines, France). The resulting plasmids were digested with *Eco*RI and *Xho*I. The fragments of interest were purified by preparative agarose gel electrophoresis, and were subsequently inserted between the *Eco*RI and *Xho*I sites of the pCMV-B10 expression vector. *Env* gene fragment nucleotides 1642–1683 and 1963–2010, obtained by hybridization with complementary oligonucleotides (Genset, Paris, France), were subcloned between the *Eco*RI and *Xho*I sites of the pCMV-B10 expression vector. We checked for in-frame insertion within the preS2 sequence of the HBV envelope gene by restriction mapping and nucleotide sequencing of the plasmids.

We used pCMV-S2.S (Michel et al., 1995), which encodes the small (S) and middle (preS2 + S) proteins of the HBV envelope (ayw subtype) under the control of the CMV promoter as a control DNA.

Expression of hybrid vectors

In vitro expression of the hybrid vectors encoding fusion proteins. The expression of the different proteins resulting from the fusion of the inserted antigenic domains with the HBsAg was verified by in vitro transcription/translation (TNT® T7 Coupled Reticulocyte Lysate System, Promega, France). The proteins synthesized from the different hybrid constructions were analyzed by electrophoresis on polyacrylamide gel (15%).

Transient transfections of mouse fibroblast with hybrid expression vectors encoding fusion proteins. Mouse fibroblast L cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 UI/ml), streptomycin (100 µg/ml), L-glutamine (2 mM) (Gibco BRL, Cergy Pontoise, France) and 10% FCS. For transfection, 3×10^5 cells were cultured in 12-well tissue culture plates. At the day of transfection, the cells reached 70% of confluency. Cells were washed twice with serum-free DMEM before transfection. Plasmid DNA transient transfections were performed using DEAE Dextran (10 mg/ml, Pharmacia Fine Chemicals) as described (Lopata et al., 1984). Each transfection experiment was performed in duplicate. At different times after transfection, supernatants were harvested for quantification of secreted HBsAg particles using a commercial ELISA kit (Monolisa Ag HBs; Biorad, Marnes la Coquette, France). We checked if the synthesized HBsAg particles detected by Monolisa also expressed HIV proteins in hybrid particles. This was performed by a specific ELISA at day 35 of culture of pCMV.B10.Env IV transfected fibroblast cells, consisting of the capture of Env IV/HBsAg particles using 2F5 monoclonal antibody and subsequent revelation using anti-HBs antibodies.

Mice and DNA-based immunization

Female HLA-A2 ($HHD^{+/+}$ $\beta 2m^{-/-}$ $D^{b-/-}$) transgenic mice (Pascolo et al., 1997) and H-2^d BALB/c mice (Iffa Credo, France) used for immunizations were 6–8 weeks old. Mice were used for immunogenicity studies of the recombinant pCMV.B10 plasmids encoding SIV Gag or HIV-1 Env epitopes, pCI-Gag or pCI-Env encoding full length proteins, as well as pCMV.S2S as control (Michel et al., 1995). All DNA vectors used for immunization were purified with Endofree Qiagen kits (Hilden, Germany). DNAs were injected into regenerating (i.e. cardiotoxin treated) *tibialis anterior* muscles as previously described (Mancini et al., 1996). Each muscle was injected with 50 µl of DNA at 1 mg/ml in PBS, such that each animal received a total of 100 µg of DNA. The i.m. injections were carried out under anesthesia (sodium pentobarbital, 75 mg/kg, i.p.). All mice were bred in the animal facility of the Pasteur Institute according to the recommendations of the ethics committee.

Cytotoxic assay

Immunized mice were sacrificed and spleens were removed 2 weeks after DNA-based immunization. Splenocytes were cultured (10^7 cells/well in 24-well plate) in 2 ml of Minimum Essential Medium (α-MEM, Gibco, Cergy Pontoise, France) supplemented with 10 mM Hepes, non-essential amino acids, 1 mM sodium pyruvate, antibiotics, glutamine (Gibco BRL, Cergy Pontoise, France), 0.05 mM β-mercaptoethanol, and 10% fetal calf serum (Myoclone, Gibco BRL).

Splenocytes from HHD immunized mice were stimulated for 7 days with peptide-loaded LPS-blast cells as APCs at an effector-presenting cell ratio of 1:1 (Loirat et al., 2000). The specific activity of effector cells was tested against HHD-transfected target cells (RMA-S HHD cell line) (Pascolo et al., 1997) pulsed with 1 μg/ml of Env HIV-1 (120–128) or HBs (348–357) HLA-A*0201 peptides.

Splenocytes from H-2^d BALB/c immunized mice were stimulated with 1 μg/ml of the various H-2^d-restricted SIV peptides derived from the Gag protein. The target cells were H-2^d murine mastocytoma cells (P815) pulsed with SIV Gag peptides (15 μg/ml), or P815 cells infected with a recombinant vaccinia virus encoding the SIV mac 239 Gag protein at a multiplicity of infection (MOI) of 20/1. Unpulsed P815 cells or wild-type vaccinia virus infected cells were used as control.

Target cells were labeled with ^{51}Cr (3.7 MBq/10^6 cells, Amersham, UK) and mixed with effector cells at different effector to target cell ratios. After a 4 h incubation at 37 °C, 50 μl of supernatants were collected and counted on a beta counter as described (Buseyne et al., 1996). Spontaneous and maximum releases were determined from targets incubated with either medium alone or lysis buffer (5% Triton X-100, 1% SDS). Percentage of specific release was calculated as (experimental release−spontaneous release)/(maximum release−spontaneous release) × 100. The specific lysis was determined for each point in triplicate.

ELISPOT assay

IFN-γ releasing cells were quantified after peptide or vaccinia virus infected cell stimulation by cytokine-specific enzyme-linked immunospot assay (ELISPOT) as described (Loirat et al., 2000; Marsac et al., 2002). To test the immunogenicity of the pCMV.B10.Env I hybrid vector in HHD mice, freshly isolated splenocytes were incubated with 1 μg/ml HIV-1 Env 120–128 or HBs 348–357 peptides, or were infected with recombinant vaccinia virus encoding either the three HBV envelope proteins (rvv S1.S2.S) or the HIV-1 envelope proteins (rvv Env HIV) at a MOI of 1/1. To test the immunogenicity of pCMV.B10.Gag-SIV hybrid vectors in H-2^d BALB/c mice, splenocytes were incubated with SIV Gag peptides (1 μg/ml) or infected with rvv S1.S2.S or the rvv encoding the SIV mac 239 Gag (rvv Gag SIV) proteins. Wells containing cells in culture medium or infected with wild-type vaccinia virus were used as negative controls to evaluate background level. ConA (5 μg/ml) stimulated splenocytes were used as positive controls. Revelation was performed as previously described (Marsac et al., 2002). The number of IFN-γ-secreting blue spots was counted and results were expressed as single spot forming cells (SFC) per million PBMC. Each cell population was titrated in triplicate. The number of SFC was determined using computer-assisted image analysis software (KS ELIspot, Zeiss, Munchen, Germany).

The percentage of $CD8^+$ and $CD4^+$ T cells was determined by FACS analysis of fresh splenocytes using direct staining with an anti-mouse CD8-FITC and CD4-PE antibodies (Pharmingen, San Diego, CA).

Serology

H-2^d BALB/c mice were immunized with 100 μg of hybrid DNA vector encoding pCMV.B10.Env II and pCMV.B10.Env IV in regenerating muscles, and boosted 2 weeks later with 50 μg of DNA. Blood was collected 2, 4 and 6 weeks after the first injection, and sera were assayed for anti-HBs antibodies (Malanchere-Bres et al., 2001) and for anti-V3 and anti-gp41 respective antibodies by specific ELISA. HBsAg particles (1 μg/ml), V3 (302–337) and gp41 (659–675) synthetic HIV-1 peptides (2.5 μg/ml) were used as the solid phase after incubating in bicarbonate buffer pH 9.6 supplemented with 10% fetal calf serum for 1 h at 37 °C and overnight at 4 °C. After blocking 1 h at 37 °C with PBS containig 0.1% Tween 20 (PBST) supplemented with 10% fetal calf serum and four washes with PBST, serial dilutions of sera were added. After extensive washing, the bound antibodies were detected with anti-mouse Ig (total IgG) labeled with horseradish peroxidase (Amersham, Little Chalfont, United Kingdom). Antibody titers were determined by the serial end-point dilution method, defined as the highest serum dilution that resulted in an absorbance value (OD 492 nm) two times greater than that of control serum with a cutoff value of 0.05. Mouse sera were tested individually, and titers were expressed as the mean of six determinations. Serum dilutions below 1/100 were considered negative.

Results

Hybrid DNA expression vectors

We used DNA vectors encoding large antigenic fragments (up to 120 amino acids) derived from the HIV Env and SIV Gag proteins, carried by a highly

antigenic molecule, the HBsAg. All the expected HIV or SIV hybrid proteins encoded by the recombinant plasmids were correctly expressed when assessed using the in vitro transcription/translation kit (data not shown).

Quantification of HBsAg particle production in supernatants of transfected murine fibroblast cell line was performed using Monolisa. Fig. 2 shows kinetics of in vitro HBsAg secretion from cell culture supernatants taken at three time points. Transfections with pCMV.B10.Gag I, II and III gave rise to the secretion of HBsAg particles at various levels (30, 6 and 1.7 ng/ml, respectively, at day 21; Fig. 2A). Transfection with pCMV.B10.Gag III gave rise to the lowest amount of HBsAg, probably due to the very large size of the hybrid Gag III/HBsAg protein expressed and/or to masking of HBsAg epitopes by the Gag domain. Therefore, we measured the intracellular production of HBsAg particles after lysis of cells transfected with the pCMV.B10.Gag III construct and low levels of Gag III/HBsAg particles were observed (2 ng/ml at day 21). Transfections with pCMV.B10.Env I and II gave rise to small amounts of HBsAg particle production (2.8 ng/ml for both plasmids at day 21). Transfections with pCMV.B10.Env III gave rise to the highest amount of particles in supernatants at day 15 (66.8 ng/ml; Fig. 2B). Transfections with pCMV.B10.Env IV gave rise to a maximum particle production at day 35 (34.8 ng/ml). Transfections of mouse fibroblast cell line with pCMV-S2.S DNA served as a positive control, resulting in a very high production of HBsAg particles in supernatants (Fig. 2C, 410 ng/ml at day 21). Hybrid particles were 10–100 fold less efficiently secreted or detected than native particles.

Furthermore, using monoclonal antibody 2F5 in a sandwich ELISA assay, it was shown that transfection with pCMV.B10.Env IV plasmid resulted in secretion of hybrid HBsAg particles presenting ELDKWA determinant on its surface (not shown). Taking into account the proteins detected after in vitro translation with all the hybrid vector constructions, it can be assumed that all the constructions gave rise to hybrid particles.

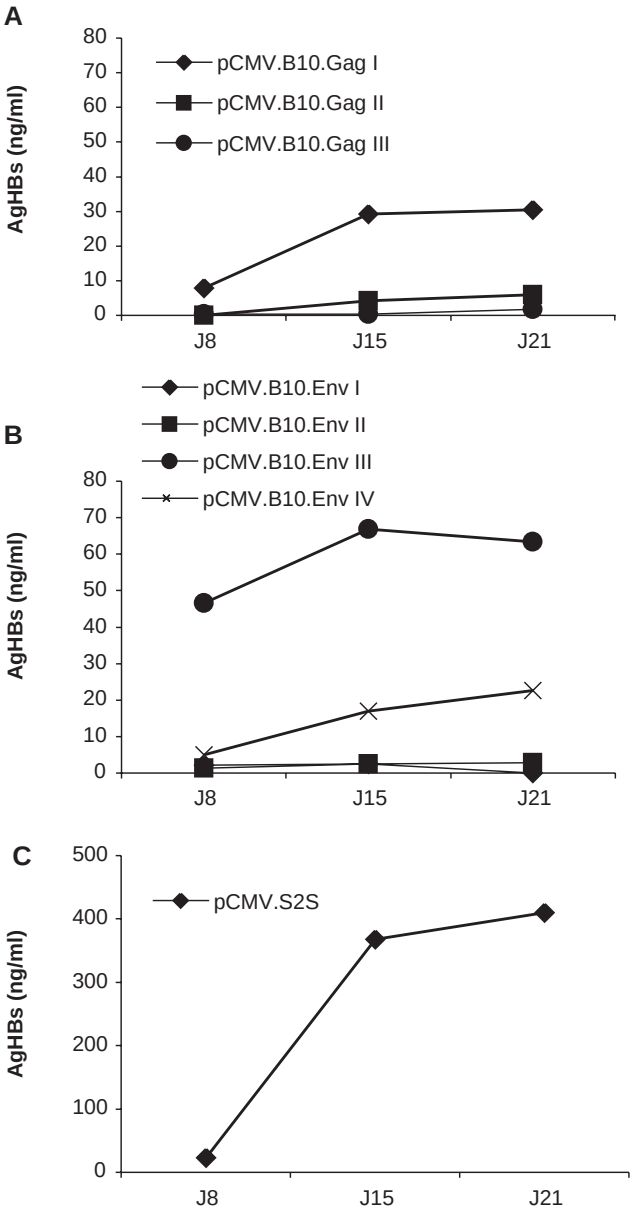


Fig. 2. Quantification of HBsAg particles in supernatants of transiently transfected murine fibroblast culture. Mouse L cells were transfected with pCMV.B10.Gag I, II, III (A) or pCMV.B10.Env I, II, III, IV (B) hybrid DNA vectors. Supernatants were harvested at three time points for quantification of secreted HBsAg particles (ng/ml) using a commercial ELISA kit. Control DNA pCMV.S2S (C) encoding HBV small and middle HBV proteins that self-assemble into particles carrying HBsAg was used as positive control. Note that the scale for antigen concentration is not the same in panel C compared to panels A and B.

Humoral response in DNA-immunized BALB/c mice

Hybrid plasmids encoding B-cell epitopes of the HIV-1 envelope were tested for their capacities to induce a humoral response in mice. Mice injected with plasmid DNAs pCMV.B10.Env II and IV produced antibodies specific to HBsAg that were detected 2 weeks after DNA injection and increased to peak titers of 4×10^4 6 weeks after the first DNA injection (Fig. 3A). We also determined the kinetics of specific anti HIV-1 envelope antibodies in the sera of the injected mice. The fine specificity of the antibodies was determined using peptides in an ELISA assay (Fig. 3B). The peptide encompassing residues 302–337 of HIV-1 Lai detected anti-V3 specific antibodies in the sera of each mouse 2 weeks after injection of pCMV.B10.Env II DNA. Titers increased slightly during the weeks following booster

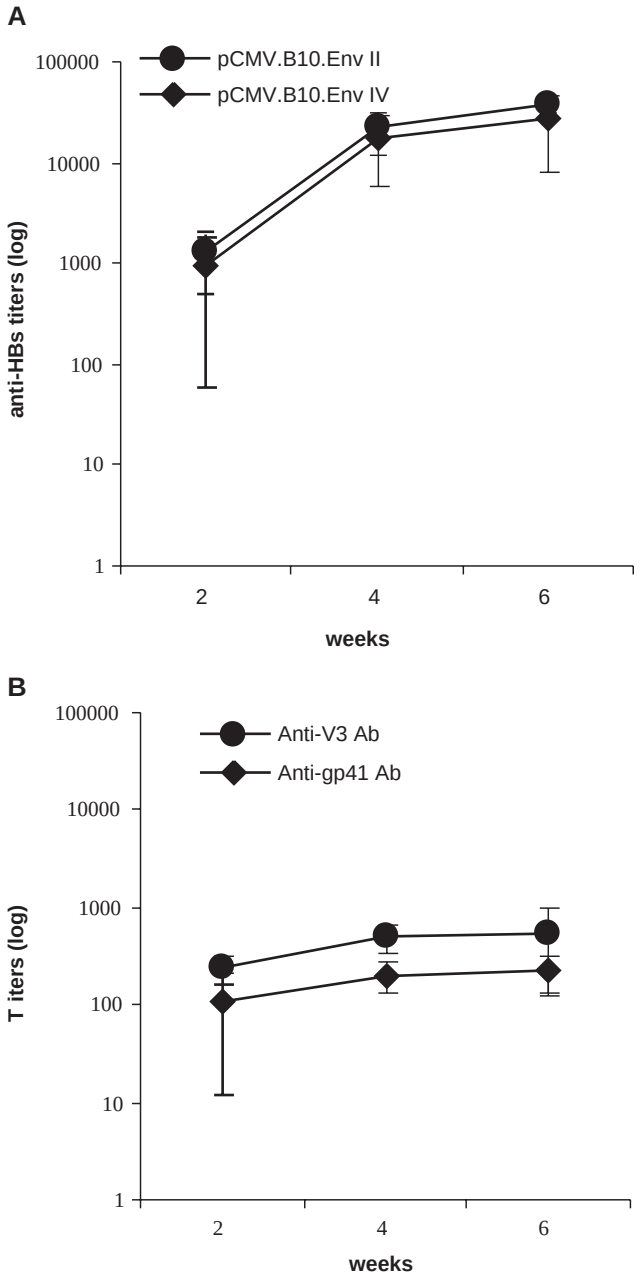


Fig. 3. Kinetics of anti-HBs (A), anti-V3 and anti-gp41 (B) antibody production. Six BALB/c mice were injected with expression vectors pCMV.B10.Env II (black circles) or pCMV.B10.Env IV (black diamonds). Sera were collected every two weeks after a booster injection of DNA given 2 weeks post prime. Each time point represents the mean titer for the group of six mice and error bars represent the standard error.

injection given at week 2. The peptide encompassing residues 659–675 including the ELDKWA motif of the highly conserved gp41 region detected specific anti-gp41 antibodies in mice injected with pCMV.B10.Env IV DNA, 2 weeks after injection. These results indicate that hybrid vectors coding for HIV-1 envelope B-cell epitopes induced anti-HBV and anti-HIV humoral responses in mice.

CTL activity in hybrid vector DNA-immunized BALB/c mice

To evaluate the ability of DNA encoding hybrid HBsAg particles carrying Gag epitopes to elicit a specific CTL response, groups of four BALB/c mice were immunized with 100 μg of each hybrid plasmid (encoding SIV Gag I, II, III domains) injected into regenerating muscles. Spleen cells were harvested 2 weeks after immunization, stimulated in vitro for 5–7 days with various SIV Gag peptides that contained potential H-2^d-restricted-epitopes and tested with a ^{51}Cr release assay.

Immunizations with pCMV.B10.Gag I (AA 165–195) hybrid vector induced anti-SIV-Gag and anti-HBV CTL responses in a group of four BALB/c mice. However, splenocytes restimulated with HBV-S 28–39 peptide not only lysed HBV-specific targets but also P815 loaded with SIV-Gag peptide (166–185). The same cross activation was noted with splenocytes stimulated with the SIV-Gag peptide that lysed equally well P815 target cells loaded with HBV-S or with SIV-Gag peptides (data not shown). This cross-reactive CTL response could be attributed to a by-stander activation of T cells due to cytokines secretion.

Immunizations with pCMV.B10.Gag II (AA 246–282) hybrid vector did not activate any specific CTL response in mice, using as effector cells splenocytes stimulated in vitro either with Gag 247–265 or with Gag 256–275 SIV peptides and P815 loaded with the same Gag peptides or infected with recombinant vaccinia virus expressing the SIV Gag protein (rvv Gag SIV) or with wild-type vaccinia virus (rvv WT) as target cells (data not shown). By contrast, for the same pCMV.B10.Gag II-immunized animals we observed an HBV-specific CTL response after in vitro stimulation with H-2^d-restricted HBV-S 28–39 peptide, against P815 target cells loaded with the same peptide, confirming that mice had been efficiently immunized (data not shown). These results indicate that neither the 247–265 nor the 256–275 SIV-Gag peptides are able to activate in vivo-primed T-cells or that these sequences are not correctly processed when presented in the context of HBsAg in mice immunized with pCMV.B10.Gag II hybrid DNA.

Immunizations of a group of four mice with pCMV.B10.Gag III (AA 165–282) hybrid vector induced anti-Gag SIV and anti-HBV specific CTL responses 2 weeks after DNA injection. Splenic effector cells from hybrid DNA-injected mice stimulated in vitro with SIV-Gag 166-185 peptide specifically lysed P815 target cells pulsed with the same SIV-Gag peptide or infected with rvv Gag SIV in three out of four mice, but

neither P815 alone nor P815 infected with rvv WT as negative controls (Fig. 4A and B). Splenic effector cells from pCMV.B10.Gag III DNA-injected mice stimulated in vitro with SIV Gag 256–275 specifically lysed P815 target cells pulsed with the same SIV-Gag peptide or infected with rvv Gag SIV in one out of four mice, but neither P815 alone nor P815 infected with rvv WT as negative controls (data not shown). We also observed that splenocytes from four out of four mice stimulated in vitro with HBV-S peptide lysed targets coated with HBV-S 28–39 peptide (Fig. 4C). These results indicate that SIV-specific T cells recognizing two different peptides were activated in vivo when SIV-Gag domains are presented in the context of the HBsAg particles in mice immunized with pCMV.B10.Gag III hybrid DNA.

Frequencies of IFN-γ-secreting CD8$^+$ T cells after DNA-based immunization in BALB/c mice

Groups of four BALB/c mice were immunized with pCMV.B10.Gag I, II, III encoding, respectively, hybrid proteins HBs/Gag I SIV (AA 165–195), HBs/Gag II SIV (AA 246–282) and HBs/Gag III SIV (AA 165–282). The number of specific IFN-γ-secreting CD8$^+$ T cells was determined 2 weeks later by ELISPOT, after a short term (40 h) ex vivo stimulation of the splenocytes using either recombinant vaccinia virus (rvv S1.S2.S) encoding the three HBV envelope proteins or rvv Gag SIV encoding SIV Gag protein or rvv WT (Fig. 5A), or with HBV-S 28–39 or SIV-Gag 166–185, 220–228, 256–275 peptides (Fig. 5B). In mice immunized with pCMV.B10.Gag I, we only detected HBs-specific IFN-γ-secreting CD8$^+$ T cells in response to stimulations with rvv S1.S2.S or with HBV-S 28–39 peptide. Incubation of spleen cells with rvv Gag SIV or with SIV-Gag peptides did not stimulate any Gag-specific IFN-γ-secreting CD8$^+$ T cells. Mice immunized with pCMV.B10.Gag II hybrid DNA developed IFN-γ-secreting CD8$^+$ T cells specific for HBs and for SIV-Gag antigens, detected in four out of four mice after rvv S1.S2.S stimulation, and in three out of four mice after rvv Gag SIV stimulation of splenocytes (Fig. 5A). Interestingly, incubation of spleen cells with HBs 28–39 and SIV-Gag 256–275 peptides stimulated identical frequencies of HBs and Gag-specific IFN-γ-secreting CD8$^+$ T cells in all four immunized mice (Fig. 5B). Mice immunized with pCMV.B10.Gag III hybrid DNA

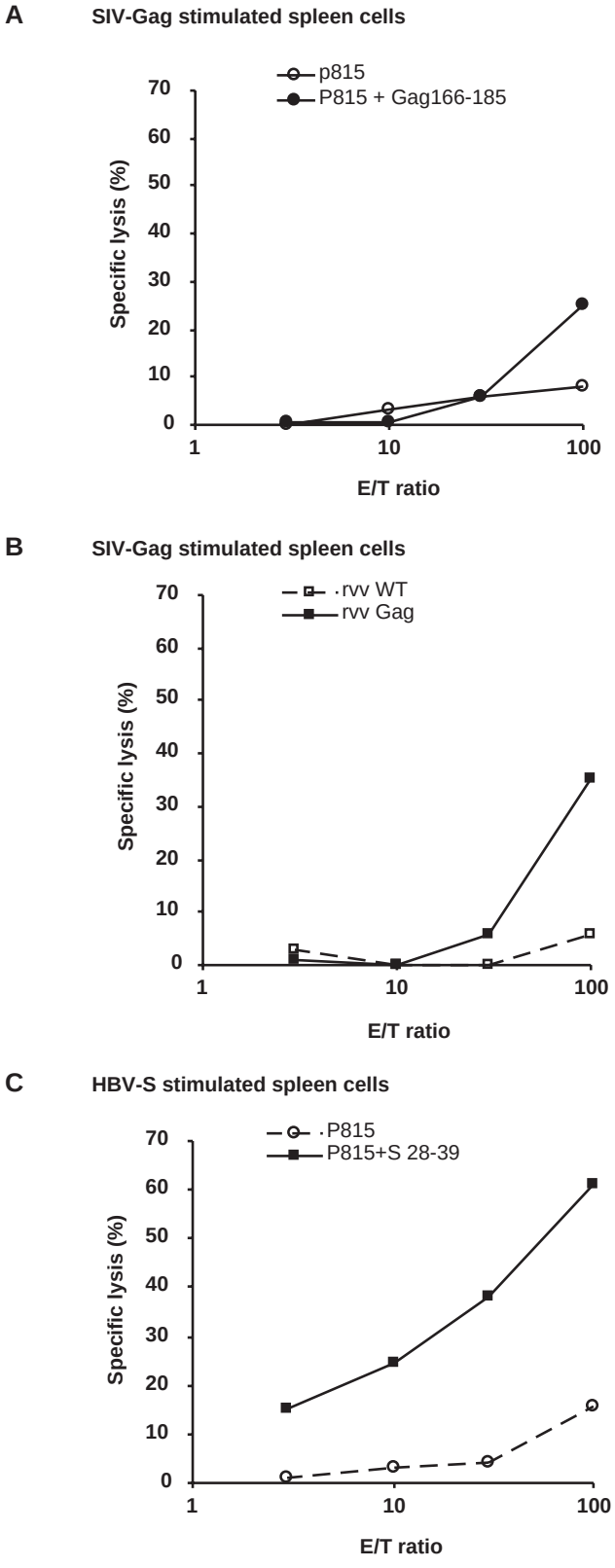
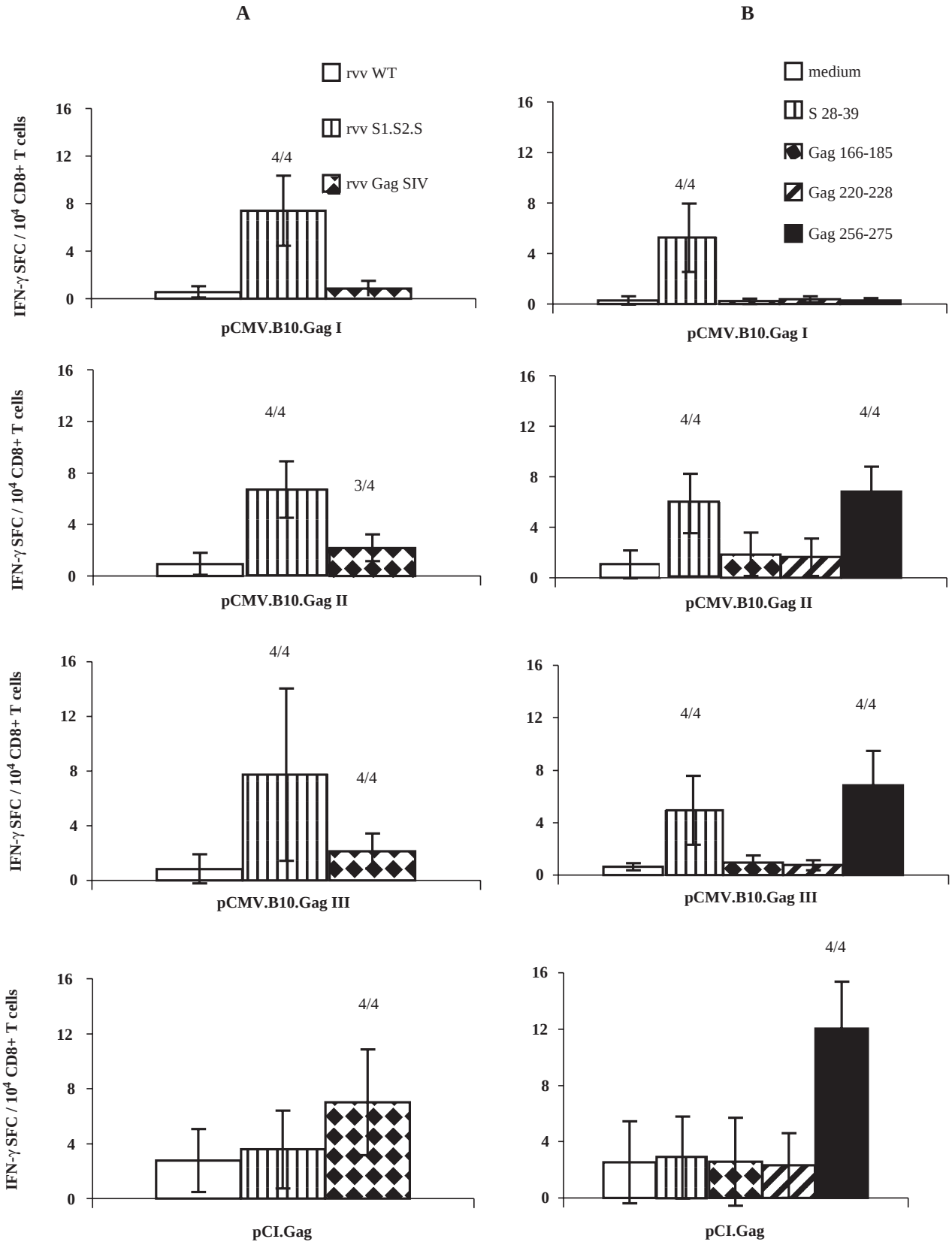


Fig. 4. Detection of CTL in BALB/c mice immunized with pCMV.B10.Gag III hybrid DNA vector. Group of four mice was immunized i.m. with 100 µg of DNA. Spleen cells were stimulated in vitro with SIV-Gag 166-185 peptide (A, B) or HBV-S 28-39 peptide (C) (1 µg/ml) for 7 days and tested for lysis of target cells. Target cells were P815 cells loaded with the same peptides (A, C) or infected with the recombinant vaccinia virus encoding the SIV mac 239 Gag protein (rvv Gag) or wild-type vaccinia virus (rvv WT) (B). Results represent the mean specific lysis for 3/4 Gag-responding mice (A, B) and for 4/4 HBs-responding mice (C).


A
B
rvv WT
rvv S1.S2.S
rvv Gag SIV
medium
S 28-39
Gag 166-185
Gag 220-228
Gag 256-275
IFN-γ SFC / 10^4 CD8+ T cells
pCMV.B10.Gag I
pCMV.B10.Gag II
pCMV.B10.Gag III
pCI.Gag
4/4
3/4

developed IFN-γ-secreting CD8$^+$ T cells specific for HBs antigens detected after rvv S1.S2.S (Fig. 5A) and HBs 28–39 peptide (Fig. 5B) stimulations. The same group of pCMV.B10.Gag III immunized mice developed IFN-γ-secreting CD8$^+$ T cells specific for SIV-Gag detected after rvv Gag stimulation but at low frequencies (Fig. 5A), and after SIV Gag 256–275 peptides stimulation at equal or greater frequencies than that observed with HBs 28–39 stimulation (Fig. 5B) in all immunized mice.

A group of four BALB/c was immunized with pCI.Gag encoding the whole Gag SIV mac239 protein. Splenocytes from pCI.Gag DNA-immunized mice secreted IFN-γ in response to stimulation with rvv Gag SIV infected cells presenting endogenously processed Gag SIV peptides, despite background measured with splenocytes stimulated with rvv S1.S2.S, rvv WT or with cells in medium only (Fig. 5A). Furthermore, mice immunized with pCI.Gag DNA displayed higher frequencies of IFN-γ-secreting CD8$^+$ T cells in response to stimulation with SIV-Gag 256–275 peptide ($12/10^4$ CD8$^+$ T cells compared to $6.8/10^4$ CD8$^+$ T cells in response to stimulation with SIV-Gag 256–275 peptide in mice immunized with both pCMV.B10.Gag II and pCMV.B10.Gag III hybrid DNA) (Fig. 5B). These results demonstrate that the Gag epitope contained within the SIV-Gag 256–275 peptide is presented after pCI.Gag-DNA or pCMV.B10.Gag II or III hybrid-DNA immunization.

CTL responses in HHD mice immunized with hybrid DNA vector

HHD-transgenic mice express human HLA-A2 but no mouse MHC class I molecules. It was described that these mice have lower expression of class I molecules and lower numbers of CD8$^+$ T cells, but they display a qualitatively normal CD8$^+$ T-cell repertoire (Pascolo et al., 1997).

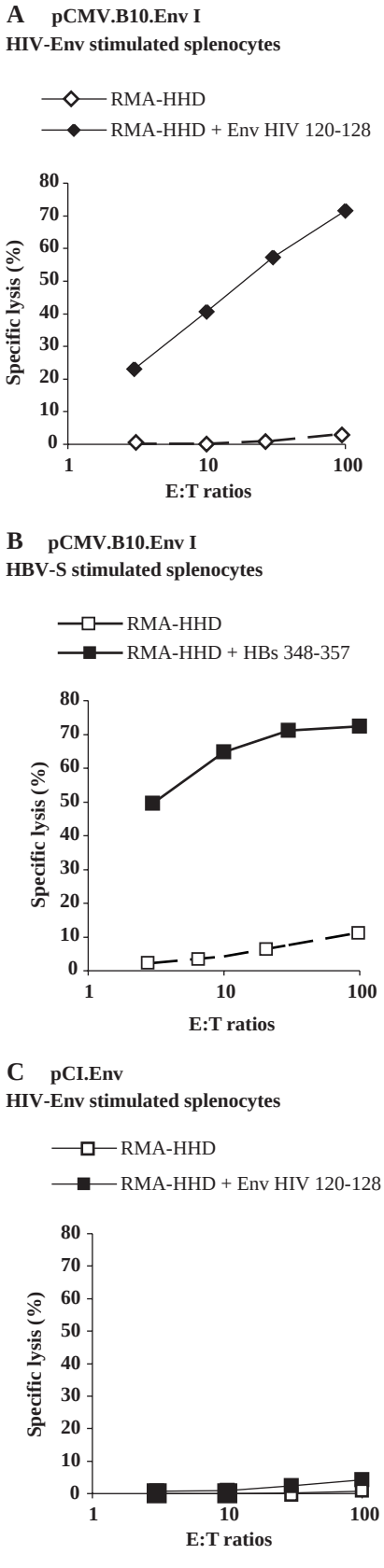
To study HLA-A2-restricted CTL response after immunization with the hybrid constructs, groups of four HHD mice were injected i.m. with pCMV.B10.Env I HIV-1 (AA 104-130). Spleens were harvested 2 weeks after DNA-based immunization, and splenocytes were stimulated in vitro with LPS blasts loaded with HBs 348–357 or gp120 HIV Env 120–128 HLA-A2 peptides. Cytotoxic activity was measured against ^{51}Cr-labeled HHD-transfected RMA-S cells loaded with the corresponding peptides. HBs 348–357 is known to be a dominant HLA-A2 epitope within the HBV envelope, inducing a strong CD8$^+$ T-cell response in HHD mice and in HBV-infected patients (Loirat et al., 2000), and HIV Env 120–128 is defined as an epitopic peptide of intermediate affinity for HLA-A2 molecule (Firat et al., 2001) in this animal model. Specific anti-HBs and anti-Env HIV-1 CTL responses were induced in three out of four pCMV.B10.Env I-injected animals (Fig. 6). At an effector to target ratio of 100/1, HBV-S and HIV-1 Env stimulated splenocytes both displayed 70% of specific lysis against target cells loaded with the corresponding peptides (Fig. 6A and B). This result indicates that the development of the anti-HBs cytotoxic response is not limiting the magnitude of the HIV-1 Env-specific CTL response and that HIV-1 Env CTL epitope is well presented in the context of HBsAg.

To better characterize the efficacy of hybrid vectors to elicit an Env-specific CTL response, we injected a group of four HHD mice i.m. with plasmid DNA pCI.Env encoding the whole HIV-1 envelope protein. Splenocytes stimulated with Env HIV 120–128 peptide and tested against HHD-transfected RMA-S cells pulsed with the same peptide did not display any cytotoxic specific activity (Fig. 6C). This suggests that the response to this epitope is subdominant when induced in the context of the whole protein. In contrast, fusion of this epitopic sequence to HBsAg does not hamper HIV-specific CTL induction.

Frequencies of IFN-γ-secreting CD8$^+$ T cells after DNA-based immunization in HHD mice

To further characterize the T-cell responses induced following DNA immunization with hybrid vectors in HHD-transgenic mice, the numbers of specific IFN-γ-secreting CD8$^+$ T cells were quantified in an IFN-γ ELISPOT assay. A group of five HHD mice was immunized with 100 µg of pCMV.B10.Env I DNA and assayed 2 weeks later for determination of the number of specific IFN-γ-secreting CD8$^+$ T cells by ELISPOT, after a short-term stimulation of the splenocytes (40 h)

Fig. 5. IFN-γ-secreting CD8$^+$ T cells after DNA-based immunization of BALB/c mice. Groups of four mice were injected i.m. with pCMV.B10.Gag I, pCMV.B10.Gag II, pCMV.B10.Gag III hybrid vectors or with pCI.Gag vector. Two weeks later the number of specific IFN-γ-secreting CD8$^+$ T cells was determined by ELISPOT. Splenocytes were infected with recombinant vaccinia virus (rvv S1.S2.S) encoding the three HBV envelope proteins or with rvv Gag SIV encoding SIV Gag protein (A, left panels) or incubated with HBs S 28-39 or with SIV Gag 166-185, 220-228, 256-275 peptides (B, right panels). Background levels were measured, respectively, after infection of splenocytes with wild-type vaccinia virus (rvv WT) or in wells containing splenocytes in medium only. The results $\pm$ SEM are given for 10^4 CD8$^+$ T cells, as determined by FACS analysis. The number of responding/tested mice is indicated at the top of each column.



with either HIV-1 Env 120–128 and HBs 348–357 HLA-A2 peptides (Fig. 7A), or infected with recombinant vaccinia virus encoding the three HBV envelope proteins (rvv S1.S2.S), the HIV-1 envelope proteins (rvv Env HIV-1) or rvv WT (Fig. 7B). The mean of $CD8^+$ T cells specific for peptide HIV-1 Env 120–128 was more than two-fold higher than that for peptide HBs 348–357 measured in 2/5 and 3/5 of the same responding mice, respectively (Fig. 7A). This result indicates that the HIV-1 Env 120–128 CTL epitope is correctly processed within the 104–130 HIV-1 domain fused to HBsAg and presented by HLA-A2 class I molecules. By contrast, we did not detect any specific $CD8^+$ T cells against target cells infected with rvv Env HIV-1 and displaying the totality of the HIV-1 envelope epitopes, whereas the frequencies of IFN-γ-secreting $CD8^+$ T cells stimulated with splenocytes infected with rvv S1.S2.S was $4.2/10^4$ of total $CD8^+$ T cells in 4/5 responding animals (Fig. 7B). Results obtained after ex vivo stimulation of pCMV.B10.Env I-primed T cells with Env-expressing rvv (Fig. 7A) suggest that HIV-1 Env 120–128 epitope is not dominant when presented to lymphocytes by recombinant vaccinia virus-infected cells.

We also immunized a group of four HHD mice with the pCI.Env plasmid encoding the whole HIV-1 envelope protein. We did not detect any IFN-γ-secreting $CD8^+$ T cells after ex vivo stimulation of splenocytes with HIV-1 Env120–128 peptide (Fig. 7A) or after infection with rvv Env HIV-1 (Fig. 7B). Stimulation with HBs 348–357 peptide or rvv WT was negative as well. These results indicate that Env I CTL epitope is better presented in the context of the hybrid HBsAg particle than after pCI.Env-DNA immunization and expression of the whole envelope protein.

Discussion

The data presented here demonstrate the efficacy of immunization with hybrid DNA constructs encoding SIV and HIV antigenic domains fused to HBsAg in inducing HBV and HIV-specific B- and T-cell responses.

Fig. 6. CTL responses after hybrid DNA immunization of HHD mice. Groups of four HHD mice were immunized i.m. either with pCMV.B10.Env I plasmid (A, B) or with the pCI.Env plasmid (C). Splenocytes from individual mice taken 2 weeks later were stimulated in vitro for 7 days with HBs 348-357 or Env HIV 120-128 HLA-A2-restricted peptides loaded on autologous LPS-blast cells. Cytotoxic activity was measured against RMA-S HHD cells loaded with the same peptides. Results represent the mean of specific lysis of the three responding mice immunized with pCMV.B10.Env I hybrid vector and the mean of four responding mice with pCI.Env-HIV-1 plasmid. E:T, effector to target ratios.

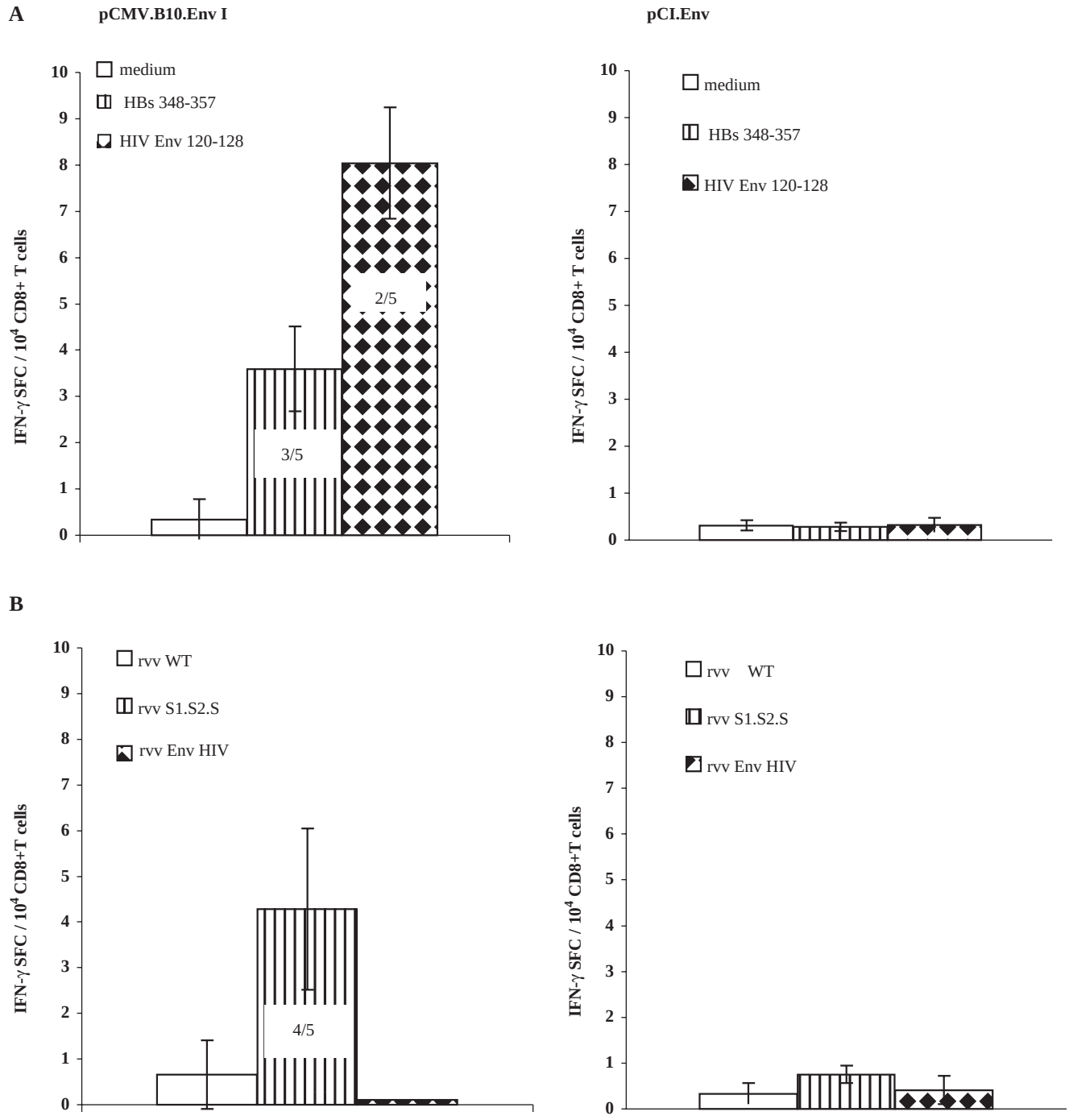


Fig. 7. IFN-γ-secreting $CD8^+$ T cells after DNA-based immunization of HHD mice. A group of five mice was injected i.m. with pCMV.B10.Env I hybrid vector (left panel) or pCI.Env plasmid (right panel). Two weeks later the number of specific IFN-γ secreting $CD8^+$ T cells was determined by ELISPOT. Splenocytes were incubated 40 h with HIV-1 Env 120-128 and HBs 348-357 HLA-A*0201-restricted peptides (A), or were infected with recombinant vaccinia virus encoding either the three HBV envelope proteins (rvv S1.S2.S) or the HIV-1 envelope proteins (rvv Env HIV) (B). Background levels were measured, respectively, in wells containing splenocytes in medium only or after infection of splenocytes with wild-type vaccinia virus (rvv WT). The ratio of responding mice is indicated in each column. The results $\pm$ SEM are given for 10^4 $CD8^+$ T cells, as determined by FACS analysis.

In our study, sequences encoding SIV-Gag and HIV-1 Env B- and T-cell epitopes have been cloned in the pCMV.B10 expression vector and further injected in BALB/c and HHD (HLA-A2 transgenic) mice. The antigenic domains fused to the HBV envelope proteins were selected for further experiments on the basis of their potential immunogenicity in infected Rhesus monkeys (Puaux et al., 2004).

The capacity of DNA immunization to induce a persistent immunity is now well established. DNA vaccines mimic live attenuated viral vaccines without any risk of pathogenicity from the viral genome (Gurunathan et al., 2000). They represent a useful approach in the development of an anti-HIV vaccine as multiple HIV-1 genes encoding B-cell and CTL epitopes can be easily manipulated (Fomsgaard et al., 1998; Firat et al., 2001; Bazhan et al., 2004). The strategy we chose here takes advantage of DNA immunization and the assembly of DNA-encoded proteins into VLPs facilitating uptake into cross-priming APCs and access to the MHC-I pathway (Bachmann et al., 1996).

We have shown that the SIV Gag III (AA 165–282) antigenic domain, presented in the context of HBsAg particle, is processed into peptides that activate a CTL response and IFN-γ-secreting T cells in vivo. Splenocytes stimulated in vitro with Gag 166–185 peptide were able to lyse rvv Gag-infected target cells expressing the whole SIV-Gag antigen, as well as Gag 166-185 peptide-loaded target cells.

Antigen-specific $CD8^+$ T cells characterized by IFN-γ production (Fig. 5) do not demonstrate any better immunogenicity of the hybrid DNA constructions compared to the original pCI.Gag plasmid in BALB/c mice. This result suggests that the concomitant anti-HBs-specific T-cell response induced with hybrid vectors may reduce the intensity of the anti-Gag-specific response in hybrid DNA-immunized mice. After internalization, the HBsAg particles follow a TAP-independent pathway in DCs and macrophages (Schirmbeck et al., 1995; Stober et al., 2002). The presentation via HBsAg VLPs involves binding of antigenic peptides derived from endocytosed HBsAg particles to empty Ld molecules in endosomal compartments and recycling to the cell surface of APCs (Schirmbeck and Reimann, 1996; Stober et al., 2002). Peptide competition for those unstable empty class I molecules can possibly occur before the complexes are stabilized by their association with β2-microglobulin. Thus, the competition of HBV and SIV antigens for intracellular processing and MHC class I molecule presentation is a possible inconvenience of these hybrid DNA constructs. Nevertheless, the ability of HBsAg particles to deliver genetically inserted heterologous antigens was largely demonstrated during the past 10 years in rodent and primate models (von Brunn et al., 1991; Schlienger et al., 1992; Lee et al., 1996; Le Borgne et al., 1998; Bojang et al., 2001; Le Borgne et al., 2001), but this is the first study using DNA inserts encoding SIV Gag epitopes. The lack of superiority of the hybrid HBsAg VLPs compared to pCI.Gag DNA to induce Gag-specific CTL responses may be correlated to intrinsic properties of the Gag protein. It is known that the HIV-1 Gag polyprotein can associate into non-infectious VLPs (Gheysen et al., 1989; Wills and Craven, 1991) and stimulate strong CTL responses in mice and other animals (Huang et al., 2001; Shiver et al., 2002). After pCI.Gag immunization, the secreted Gag protein may auto-assemble into VLPs and may be cross presented by DCs thus enhancing the anti-Gag specific CTL response.

To study the cellular response induced after immunizations with hybrid vector encoding HIV-1 envelope proteins, we chose the HLA-A2-transgenic mouse model suitable for preclinical immunogenicity studies of antigens (Woodberry et al., 1999). Recently, a pCMV.B10-based multiepitopic construct was used for DNA immunization in HHD mice and activated a long-lasting multispecific anti-HIV-1 CTL response (Firat et al., 2001). In our study, HIV-1 Env-specific CTL were generated in three out of four immunized mice (Fig. 6A), with levels of CTL lysis identical to those from HBV-specific splenocytes recognizing peptide HBs 348–357, which is known to be an immunodominant CTL epitope in this mouse model (Loirat et al., 2000). This result indicates that anti-HBV specific CTL responses do not predominate over the HIV Env CTL response and also that there is no competition between HIV Env and HBs CTL epitopes in this MHC context. Furthermore, in the same responding mice the frequencies of IFN-γ-secreting $CD8^+$ T cells were more than two-fold higher in splenocytes stimulated with Env 120–128 specific peptide compared to the frequencies of IFN-γ-secreting $CD8^+$ T cells stimulated with HBs 348–357 peptide (Fig. 7A). It was recently demonstrated in BALB/c mice that immunodominant Ld-restricted $CD8^+$ T-cell response to S 28–39 epitope down-modulate concomitant response to other HBsAg-derived epitopes (Schirmbeck et al., 2002). For establishing this immunodominance hierarchy the competing epitopes may have to be present on the same protein. This could explain why $H\text{-}2^b$-restricted Gag-specific response is down-modulated in the context of the Gag-HBsAg fusion protein. In contrast, in the context of HLA-A2-transgenic mice background, HBs-derived HLA-A2-restricted epitope (348–357) does not dominate over the HIV Env-derived epitope (120–128).

HIV infection elicits antibodies directed against several regions of the gp120 and gp41 envelope glycoproteins. The human monoclonal antibody 2F5 has broad HIV-1 neutralizing activity and binds to a conserved linear epitope ELDKWA within the gp41 glycoprotein (Muster et al., 1993). Neutralizing antibody response against this epitope was rarely induced in vivo (Coeffier et al., 2000; Joyce et al., 2002). Immunizations of mice with hybrid vectors encoding gp41 B-cell epitope (Env IV, AA 655–670) induced a humoral response in BALB/c mice after two DNA injections. The titers obtained were modest but this result suggests that this vaccine model could be a candidate designed to elicit a 2F5-like immune response.

Recent results obtained after immunization of Rhesus monkeys using the hybrid SIV-HIV/HBsAg-encoding DNA have shown that this strategy is a potent approach to improve immunogenicity of inserted heterologous sequences, regarding both the humoral and T-cell responses in animals with a completely unknown genetic background (Puaux et al., 2004).

Acknowledgments

This work was supported by the *Agence Nationale de Recherches sur le SIDA* (ANRS) and *Ensemble contre le Sida* (Sidaction). D. Marsac and A.-L. Puaux were supported by a fellowship from ANRS. We would like to thank Dr. François Lemonnier for providing HLA-A2 transgenic mice, A. Habel for providing pCI.Gag and pCI.Env plasmids. We also thank Delphine Loirat for technical advice and helpful discussions.

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