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Antigenic Properties of Recombinant Envelope Glycoproteins Derived from T-Cell-Line-Adapted Isolates or Primary Human Immunodeficiency Virus Isolates and Their Relationship to Immunogenicity

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The native envelope glycoproteins of primary HIV-1 virions have weaker antigenicity than do T-cell laboratory-adapted (TCLA) viruses. These antigenic properties require further evaluation if recombinant envelope glycoproteins are produced as part of a vaccine strategy. In this study, we compared the antigenicity of recombinant envelope glycoproteins derived from three primary isolates (PI) (HIV-1_{BX08}, HIV-1_{CHA}, and HIV-1_{133}) and two TCLA viruses (HIV-1_{HXB2} and HIV-1_{MN}) produced using the Semliki Forest virus (SFV) system. This analysis was performed by radioimmunoprecipitation assays and flow cytometry. The results suggest that the SFV produces envelope glycoproteins with features in common with the envelopes found in naturally occurring virions. In particular, the PI envelopes had weak heterogeneous antigenic properties. However, the cytometric analysis also showed that there was less envelope glycoprotein on the cell surface for the PI envelopes than for those of TCLA viruses, suggesting differences in their intracellular trafficking. The immunogenic properties of the various envelope glycoproteins were evaluated in mice using recombinant SFV particles as vaccine vectors. The PI envelopes were less immunogenic than the TCLA envelopes, probably due to both their low antigenicity and cell surface expression level. Thus, it may be difficult to design an effective vaccine based on native recombinant PI envelopes.

INTRODUCTION

The viral envelope glycoproteins are the main targets of the various antibody-based vaccine approaches for protecting individuals against human immunodeficiency virus type 1 (HIV-1) infection. In their native conformation, these envelope glycoproteins (gp) form oligomeric spikes displayed on the virions or on infected cell membranes (Earl *et al.,* 1990). Recent physical and crystallographic data have suggested that the spike has a trimeric organization, consisting of an external envelope glycoprotein (gp120) noncovalently associated with a subunit of a transmembrane envelope glycoprotein (gp41) trimeric complex (Chan *et al.,* 1997; Weissenhorn *et al.,* 1997; Wyat *et al.,* 1998). Such oligomeric envelope complexes have specific antigenic properties, and most of the epitopes present on monomeric envelope molecules are not available on oligomeric complexes (Burton, 1997; Earl *et al.,* 1994; Fouts *et al.,* 1997; Moore *et al.,* 1995). One determinant factor in vaccine design is eliciting antibodies that bind well to these native, oligomeric envelope complexes. Thus, vaccine strategies in which the native envelope complexes are presented to the immune system are now favored.

Another complicating factor in HIV vaccine design is raising antibodies against the viruses involved in transmission in humans, the primary viruses. Most primary viruses isolated from HIV-1-infected humans are macrophage-tropic. These primary isolates (PI) differ from T-cell tropic viruses and their T-cell laboratory-adapted variants (TCLA) in terms of the coreceptor used for virus entry. The initial steps in virus entry involve the specific binding of gp120 to the cell surface CD4 receptor and an interaction with a chemokine receptor, principally CCR5 or CXCR4 for HIV-1, which serves as coreceptor. PI use CCR5 as a coreceptor (R5 isolates), whereas T-cell tropic and TCLA viruses (X4 and X4R5 isolates) are able to use other chemokine receptors such as CXCR4 (Alkhatib *et al.,* 1996; Choe *et al.,* 1996; Feng *et al.,* 1996; Simmons *et al.,* 1996). In addition to their specific coreceptor usage, PI also have unique antigenic properties. Very little of their envelope spike surface is accessible to antibodies specific for TCLA viruses (Burton, 1997). In particular, two major epitopes present on TCLA virus envelopes, the CD4 binding domain and the V3 loop, are not very accessible and, consequently, most anti-CD4 binding site antibodies and anti-V3 loop antibodies recognize PI en-

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velopes inefficiently (Beddows *et al.,* 1998; Bou-Habib *et al.,* 1994; Moore *et al.,* 1995; Spenlehauer *et al.,* 1998). The exposure of epitopes such as the V3 loop on TCLA viruses probably reflects optimization of the virus–cell interaction in the absence of the selective pressure encountered *in vivo* by the primary viruses. Only two gp120 epitopes and one gp41 epitope have been shown to be clearly accessible on PI envelopes. These epitopes are recognized by the human monoclonal antibodies (mAbs) IgG1b12, 2G12, and 2F5, respectively (Fouts *et al.,* 1997; Trkola *et al.,* 1995). The high level of glycosylation of gp120 and the occlusion by variable loops are often put forward as reasons for the inaccessibility of epitopes on the native envelope of PI (Bou-Habib *et al.,* 1994; Reiter *et al.,* 1998; Wyat *et al.,* 1998).

Thus far, research has focused on the antigenicity of envelopes from TCLA viruses, which clearly differs from that of PI envelopes. The challenge of obtaining an immunogen that induces antibodies against the mature oligomeric envelopes of PI remains. Meeting this challenge requires better characterization of the antigenic and immunogenic properties of these envelopes.

Vaccine strategies involving the presentation of mature oligomeric envelopes *in vivo* are promising because they should mimic the early phases of interaction between naturally occurring viruses and their hosts. Thus, priority is now given to vaccine approaches based on live attenuated viruses, recombinant viruses expressing the HIV envelope, genetic vaccines, and purified oligomeric glycoproteins (Girard *et al.,* 1997; Richmond *et al.,* 1998; Robert-Guroff *et al.,* 1998; Shibata *et al.,* 1997; VanCott *et al.,* 1997).

In this work, we investigated the antigenic and immunogenic properties of envelope glycoproteins derived from three HIV-1 PI and two TCLA viruses. These envelopes were produced *in vitro* and *in vivo* using recombinant Semliki Forest virus (rSFV) RNA and rSFV particles, respectively (Berglund *et al.,* 1993; Liljeström and Garoff, 1991; Zhou *et al.,* 1994). Our main objective was to assess whether the antigenic properties described as specific for PI envelopes by comparison with TCLA virus envelopes would also be found if envelopes were produced using an expression system such as recombinant SFV. The SFV system was used for this study because it has been shown to be one of the most promising vaccine strategies based on recombinant viruses (Brand *et al.,* 1998; Mossman *et al.,* 1996).

For antigenic characterization of the envelopes, we focused on the presentation of neutralizing epitopes corresponding to the human neutralizing mAbs F105, IgG1b12, 447-52D, and 2F5 and we used assays such as radioimmunoprecipitation and flow cytometry, which respect as much as possible the native conformation of the envelopes (Conley *et al.,* 1994; Fouts *et al.,* 1997; Roben *et al.,* 1994; Thali *et al.,* 1991; Trkola *et al.,* 1995). We also evaluated the specificity of immune responses in mice inoculated with rSFV particles expressing each gp160 separately or immunized with a mixture of rSFV particles expressing the five different gp160s, to assess whether the simultaneous presentation of different envelopes *in vivo* could broaden the specificity of the immune response.

RESULTS

Radioimmunoprecipitation assay (RIPA) analysis of the recombinant envelope glycoproteins

The antigenic properties of the native solubilized recombinant envelope glycoproteins from the two HIV-1 TCLA strains (HIV-1_{HXB2} and HIV-1_{MN}) and the three HIV-1 PI (HIV-1_{BX08}, HIV-1_{CHA}, HIV-1_{133}) were analyzed by RIPA. The various envelope glycoproteins were produced by electroporating BHK-21 cells with rSFV RNAs. The glycoproteins present in cell lysates and supernatants were analyzed using a pool of sera from HIV-1-infected individuals and human neutralizing mAbs F105, IgG1b12, 447-52D, and 2F5. Both full-length gp160 and the processed form, gp120, were immunoprecipitated from all cell lysates and culture supernatants by the human sera and F105 (Figs. 1A and 1B). However, the amount of gp120 immunoprecipitated from cell supernatants was significantly lower for CHA than for envelopes of the other four strains. Neither the gp160 precursor nor the mature gp120 of HIV-1_{133} was bound by IgG1b12. In contrast, the corresponding epitope was present and accessible on the envelope precursors of the other four strains, but was not detectable on the gp120 of HIV-1_{BX08} (Figs. 1A and 1B). The mAb 447-52D and 2F5 immunoprecipitated all the gp160s, except the $gp160_{CHA}$, from the cell lysates (Fig. 1C). The results obtained with the human sera and F105 suggest that all five envelope glycoproteins were processed and correctly folded, consistent with previous work, also based on the SFV system (Brand *et al.,* 1998; Paul *et al.,* 1993). $Gp120_{CHA}$ was apparently shedded more slowly than the gp120s of the other four envelopes although a kinetic analysis should be performed to confirm this observation. As the SFV vector constructs were similar for all the envelopes, the lower shedding observed for the $gp120_{CHA}$ may be due to a slower processing of this envelope, which remains to date unexplained. Indeed, the sequence of the cleavage site was checked and the basic tetrapeptide, REKR, was present.

There were clear antigenic differences between the recombinant envelopes. The gp160s of both TCLA strains ($gp160_{HXB2}$ and $gp160_{MN}$) and only one envelope glycoprotein from the three primary isolates, $gp160_{BX08}$, presented all the epitopes recognized by the three neutralizing mAbs IgG1b12, 447-52D, and 2F5 (Figs. 1A, 1B, and 1C). The IgG1b12 conformational epitope located in

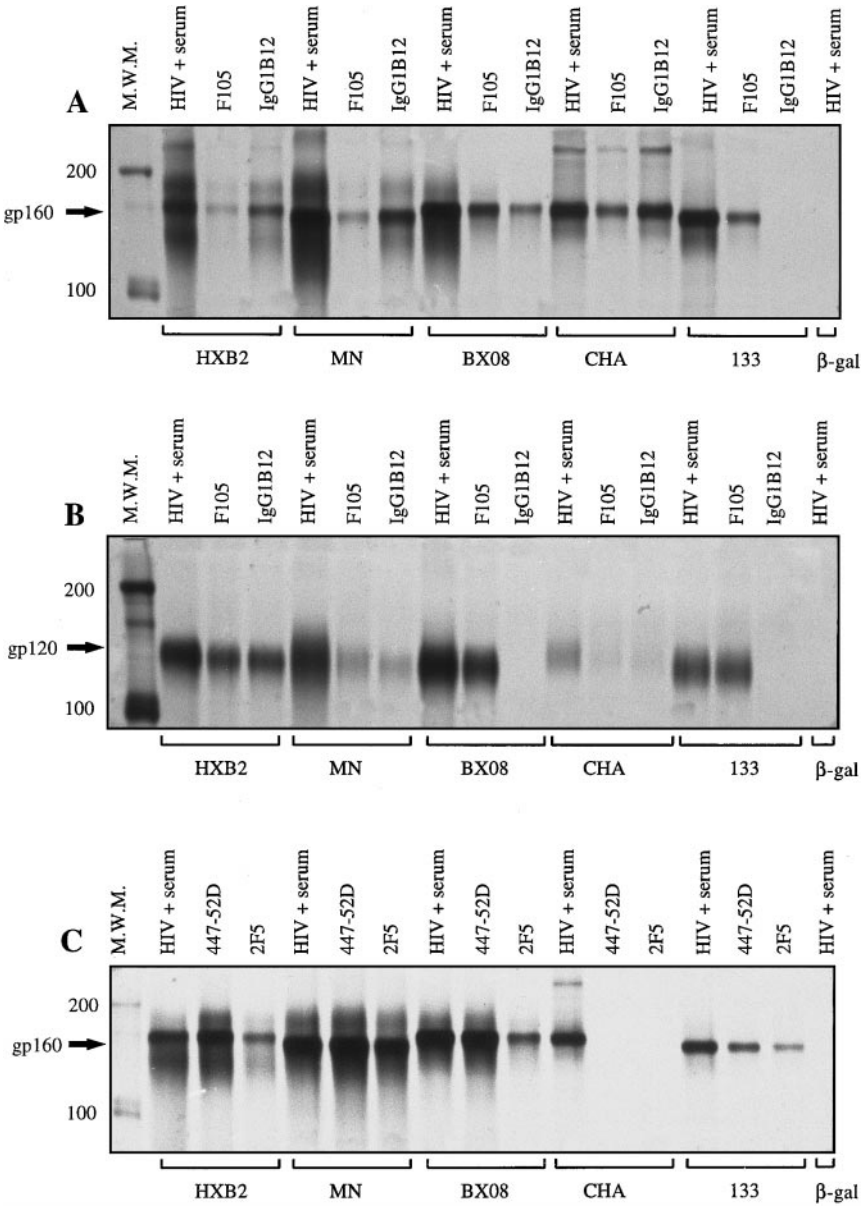

FIG. 1. Precipitation of HIV-1_{HXB2}-, HIV-1_{MN}-, HIV-1_{BX08}-, HIV-1_{CHA}-, and HIV-1_{133}-recombinant envelope glycoproteins by a pool of sera from HIV-1-infected patients (HIV + serum) and by the F105, IgG1b12, 447-52D, or 2F5 monoclonal antibodies. The envelope glycoproteins were produced by transfection of BHK-21 cells with the various rRNAs and were metabolically labeled with [^{35}S]cysteine before immunoprecipitation (see Materials and Methods). (A, C) Precipitation of gp160 from BHK-21 cell lysates; (B) precipitation of gp120 from culture supernatants. M.W.M., molecular weight marker.

the CD4 binding site region of gp120 was not detected on $gp160_{133}$ or on $gp120_{BX08}$ and $gp120_{133}$. The mAbs 447-52D and 2F5 are specific for the linear epitopes GPxR, at the center of the V3 loop in gp120, and ELDKWA, in the ectodomain of gp41, respectively (Conley *et al.,* 1994; Muster *et al.,* 1993). Only the envelope of HIV-1_{CHA}, which has different amino acid sequences for these two linear epitopes, was not recognized by 447-52D and 2F5. $Gp160_{CHA}$ bears the sequences APGR at the tip of the V3 loop and ELDTWA in the gp41 ectodomain.

Flow cytometry analysis of the native recombinant envelope glycoproteins

The cell surface expression and the antigenic properties of five native envelope glycoproteins were analyzed by flow cytometry. The amount of each envelope glycoprotein produced by BHK-21 or Vero cells was determined using a pool of sera from HIV-1-infected individuals (Fig. 2). There were major differences between the amounts of TCLA- and PI-derived glycoproteins detected on the cell surface. Indeed, the histo-

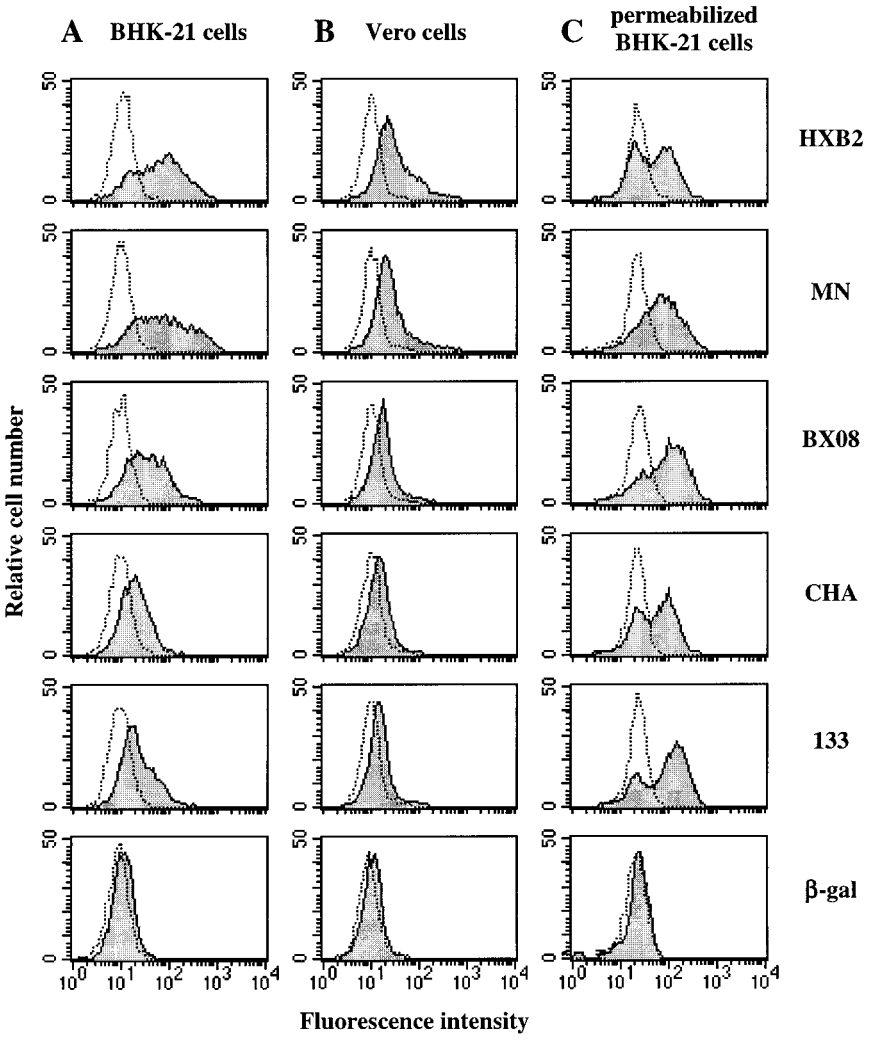

FIG. 2. Binding of antibodies present in a pool of sera from HIV-1-infected patients to HIV-1_{HXB2}, HIV-1_{MN}, HIV-1_{BX08}, HIV-1_{CHA}, and HIV-1_{133} native envelope complexes, analyzed by flow cytometry. Data are plotted as fluorescence intensity (*x*-axis) versus relative cell number (*y*-axis). In each graph, the gray area represents transfected or infected cells and the dotted line represents the mock-transformed cells (untransfected BHK-21 cells or uninfected Vero cells). (A) BHK-21 cells transfected with the various rRNAs, BHK-21 cells transfected with RNA.β-gal were used as a negative control; (B) Vero cells infected with the various rSFV; Vero cells infected with SFV.β-gal were used as a negative control; (C) BHK-21 cells transfected with the various rRNAs and permeabilized prior to immunostaining.

grams representing the level of expression of gp_{HXB2} or gp_{MN} on the surface of BHK-21 cells shift to the right much more strongly than do the histograms for gp_{CHA} and gp_{133} (Fig. 2A). More envelope glycoprotein was detected on the cell surface for BX08 than for the other two PI envelopes. The level of gp_{BX08} was, however, lower than that of the TCLA glycoproteins. We checked that the differences observed were not due to the host BHK-21 cells, by producing envelope glycoproteins in Vero cells. Cell surface expression was evaluated in the same way, by flow cytometry. The overall level of expression was lower than for BHK-21 cells but similar differences in cell surface expression were observed between the various envelopes (Fig. 2B). This second analysis confirmed that the amounts of glycoprotein detected on the cell surface with human sera were larger for TCLA isolates than for PI. We assessed whether these differences were due to variations in the overall cellular expression of the various envelopes by analyzing permeabilized BHK-21 cells by flow cytometry, such that intracellular glycoproteins were also detected (Fig. 2C). The levels of gp_{HXB2} and gp_{MN} detected in permeabilized BHK-21 cells were no higher than those for gp_{BX08}, gp_{CHA}, or gp_{133}. Thus, there was no apparent correlation between cell surface detection and overall cellular expression for the various recombinant proteins.

The cell surface expression and the antigenic properties of each native recombinant glycoprotein at the surface of BHK-21 cells were then further analyzed by flow

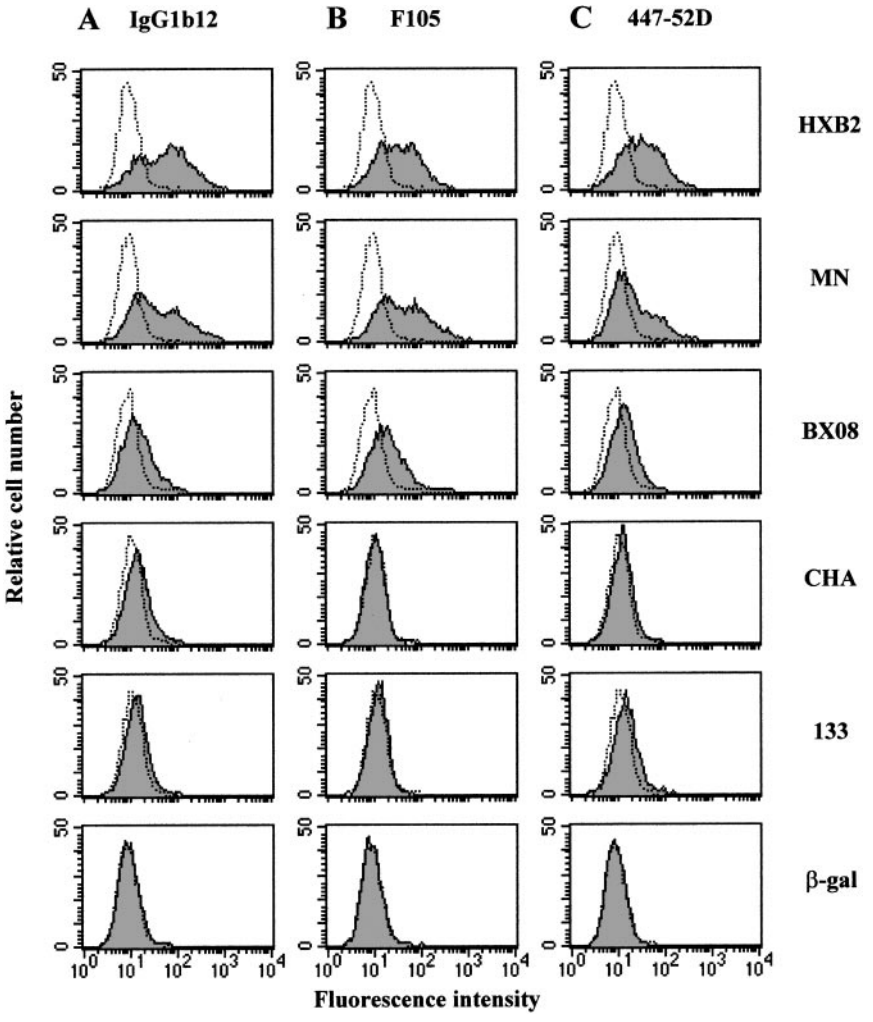

FIG. 3. Binding of human mAbs IgG1b12 (A), F105 (B), and 447-52D (C) to HIV-1_{HXB2}, HIV-1_{MN}, HIV-1_{BX08}, HIV-1_{CHA}, and HIV-1_{133} native oligomeric envelope complexes, analyzed by flow cytometry. The envelope glycoproteins were produced by transfection of BHK-21 cells with the various rRNAs. BHK-21 cells transfected with RNA.β-gal were used as a negative control. Data are plotted as fluorescence intensity (*x*-axis) versus relative cell number (*y*-axis). In each graph, the gray area represents cells expressing the HIV envelope and the dotted line curve represents the mock-transformed cells (untransfected BHK-21 cells).

cytometry using the human mAbs 2F5, F105, IgG1b12, and 447-52D. The 2F5 epitope was not detected at the surface of the cells expressing the various envelopes (data not shown). A similar result was obtained in a previous study showing that the 2F5 epitope was temperature-dependent and inaccessible at 4°C (Sattentau *et al.,* 1995). The F105-, IgG1b12-, and 447-52D-specific epitopes were much more abundant or accessible on the surface of cells producing TCLA glycoproteins than on those of cells producing PI glycoproteins (Fig. 3). Of the recombinant PI envelopes, the F105-, IgG1b12-, and 447-52D-specific epitopes were more abundant or accessible on the surface of cells producing gp_{BX08} than on those of cells producing gp_{CHA} and gp_{133}, which gave signals not significantly different from that of the β-galactosidase control (Figs. 3A, 3B, and 3C).

Immunogenicity of native envelope glycoproteins

The immunogenicity of the five native envelope glycoproteins was evaluated in BALB/c mice by immunization either with rSFV particles producing the various envelopes individually or with a mixture of SFV particles producing the different envelopes. Mice inoculated with SFV.β-gal particles were used as negative controls. Sera were collected 50 days after the first immunization and were tested by envelope-specific enzyme-linked immunosorbent assay (ELISA) for the presence of antibodies against the homologous and heterologous envelopes. Cell lysates from BHK-21 cells electroporated with the various rRNAs were used as a source of envelope glycoproteins.

We checked that equivalent amounts of envelope glycoproteins were present in the various cell lysates, by

means of controls with two pools of sera from HIV-1-infected individuals and two pools of sera from uninfected individuals (Fig. 4F).

The antibody responses obtained in mice immunized with the various immunogens were evaluated by determining antibody titers (Fig. 4). A mean A_{490} (absorbance at 490 nm) value was calculated for each group of mice, at each dilution. The results obtained showed that animals immunized with a single type of envelope tended to have the highest antibody titer against the homologous envelope, indicating a slightly strain-specific response (Fig. 4). For instance, sera from mice immunized with rSFV.HXB2 had the highest antibody titer (1:3200) against $gp160_{HXB2}$ (Fig. 4A). Similarly, sera from mice immunized with rSFV.MN, rSFV.CHA, or rSFV.133 had the highest antibody titers against $gp160_{MN}$, $gp160_{CHA}$, and $gp160_{133}$, respectively (Figs. 4B, 4D, and 4E). Antibody titers against homologous envelopes in mice immunized with rSFV producing envelopes derived from TCLA viruses were two to three dilutions higher than those obtained in mice immunized with rSFV producing envelopes from PI.

The sera collected from mice immunized with the mixture of rSFVs had the broadest specificity. Indeed, except in ELISA BX08, the antibody response of the sera from these mice was always the next strongest after that of mice immunized with the homologous envelope. Thus, the antibody response could be broadened by using a mixture of several envelopes.

The specificity of the ELISAs was checked using postimmunization sera from mice immunized with SFV.β-gal. Each serum sample was tested at a 1:100 dilution. None gave absorbance values above the cut-off point. The reactivity of postimmunization sera collected from mice immunized with SFV particles producing the various envelopes was also evaluated by ELISA using cell lysates of BHK-21 cells electroporated with RNA.β-gal. Each serum sample was tested at a 1:100 dilution. All absorbance values with this control antigen were below the cut-off point.

DISCUSSION

Several studies have shown that the oligomeric envelope complexes found on the surface in primary HIV isolates have specific antigenic properties. In particular, these envelope complexes have been described as being less antigenic than the envelope complexes found on the surface of TCLA viruses (Beddows *et al.,* 1998; Bou-Habib *et al.,* 1994; Burton, 1997; Moore *et al.,* 1995; Spenlehauer *et al.,* 1998). Strategies for obtaining an effective vaccine against HIV are now focusing on the induction of antibodies recognizing these primary virus envelope complexes, and vaccine approaches involving the presentation of native envelope complexes to the immune system are currently favored (Girard *et al.,* 1997; Richmond *et al.,* 1998; Shibata *et al.,* 1997; Van Cott *et al.,* 1997). Of the vaccine designs tested recently, approaches involving live recombinant viruses (i.e., avipoxviruses, Semliki Forest virus, adenovirus) producing HIV envelope glycoproteins used alone or in combination with recombinant soluble envelope glycoproteins in prime-boost strategies are promising (Brand *et al.,* 1998; Girard *et al.,* 1997; Robert-Guroff *et al.,* 1998). However, the different antigenic properties of PI envelope complexes relative to those of TCLA viruses, and their consequences for immunogenicity require further evaluation if the envelope is to be produced in a heterologous system such as recombinant viruses.

In this study, we evaluated the antigenic properties of recombinant envelopes from three PI (HIV-1_{BX08}, HIV-1_{CHA}, and HIV-1_{133}) and two TCLA viruses (HIV-1_{HXB2} and HIV-1_{MN}) in the SFV expression system. The antigenic properties were first analyzed qualitatively by RIPA using a pool of sera from HIV-1-infected individuals and the human neutralizing mAbs F105, IgG1b12, 447-52D, and 2F5. Each primary isolate envelope had specific antigenic properties. Indeed, all the recombinant envelope glycoproteins were bound by the human sera and by the mAb F105 but only one of the three PI envelope glycoproteins, that from HIV-1_{BX08} was recognized by all the mAbs. The RIPA performed with the mAb IgG1b12 showed that the corresponding conformational epitope was detected only on $gp160_{BX08}$ (unprocessed) and not on $gp120_{BX08}$ (processed). Thus, at least one and possibly several epitopes recognized by the neutralizing mAbs tested were missing or hidden on the recombinant envelope glycoproteins derived from PI. Furthermore, an epitope present on gp160 (unprocessed) could be hidden on gp120 (processed). Thus, the RIPA results suggested that the antigenicity of the recombinant envelope glycoproteins derived from PI was weak and heterogeneous, consistent with observations made with native virions (Beddows *et al.,* 1998; Bou-Habib *et al.,* 1994; Burton, 1997; Moore *et al.,* 1995; Spenlehauer *et al.,* 1998). The state of processing of the envelope glycoprotein should also be taken into account when examining its antigenic properties. Indeed, an epitope recognized by a neutralizing antibody such as IgG1b12 may be hidden on the protein, depending on its state.

We further evaluated the expression and antigenicity of our recombinant envelope glycoproteins: the native envelope complexes anchored to the external surface of the cellular membrane were analyzed by cytometry using a pool of sera from HIV-1-infected individuals and the human mAbs F105, IgG1b12, 447-52D, and 2F5. The results obtained with human sera showed that less envelope glycoprotein was produced on the surface of BHK-21 cells for PI than for TCLA viruses. For PI envelopes, the level of cell surface expression was very low for gp_{CHA} and gp_{133}, whereas gp_{BX08} had an intermediate

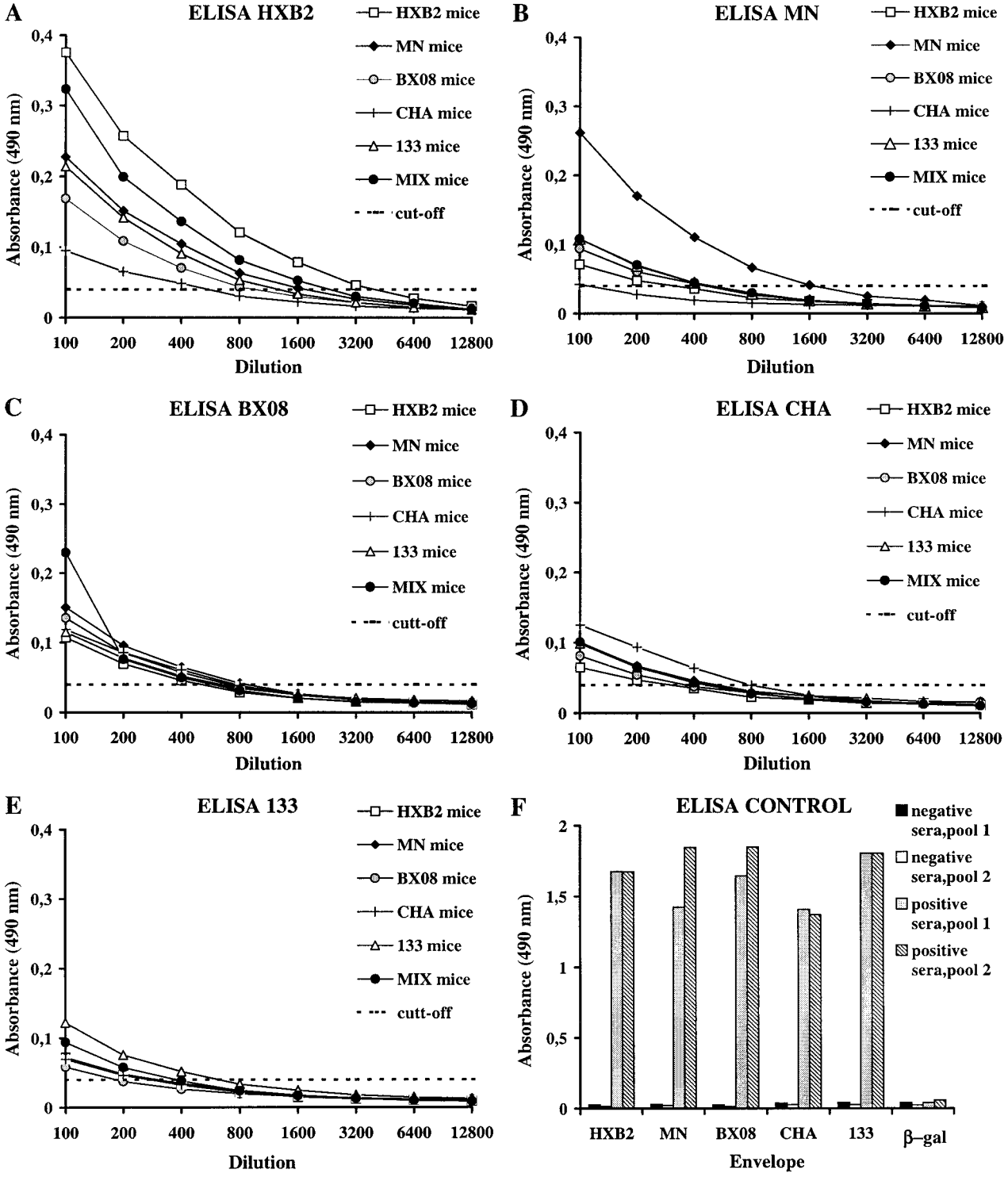

FIG. 4. Titration of anti-gp160 antibody response in sera from groups of mice immunized with rSFV.HXB2 (HXB2 mice), rSFV.MN (MN mice), rSFV.BX08 (BX08 mice), rSFV.CHA (CHA mice), rSFV.133 (133 mice), or a mixture of the five rSFV particles (MIX mice), by envelope-specific enzyme immunoassays. The envelopes captured on the solid phase were obtained from lysates of BHK-21 cells transfected with rRNA.HXB2 (A), rRNA.MN (B), rRNA.BX08 (C), rRNA.CHA (D), or rRNA.133 (E). Each group of mice is represented by a different symbol. Each point corresponds to the mean absorbance value for sera from each group at the corresponding dilution. We checked that similar amounts of envelope glycoprotein were captured on the solid phase from the various cell lysates, by carrying out an ELISA under similar conditions, using two pools of sera from HIV-1-infected patients and two pools of sera from uninfected patients (F). Sera were tested at a 1:100 dilution.

level of expression, between those of gp_{CHA} or gp_{133} and those of TCLA envelopes. These apparent differences in cell surface expression between the various envelope glycoproteins were confirmed in Vero cells, indicating that they were not related to a particular cell type. The differences observed may be due to differences in the expression levels of the various envelope glycoproteins on the cell surface or to specific antigenic properties of the primary isolate envelopes. Cytometric analysis with permeabilized BHK-21 cells showed that the overall level of expression for PI envelopes was not lower than that of TCLA virus envelopes. So, the differences observed between the expression levels of the five envelopes studied were not due to differences in the overall cellular expression between the envelope glycoproteins. Cytometric analysis with mAbs showed only weak detection of the various PI envelopes on the BHK-21 cell surface. The level of binding of mAbs to the surface of cells expressing the PI envelope gp_{BX08} was intermediate between that of cells expressing gp_{CHA} or gp_{133} and those expressing TCLA envelopes, consistent with the results obtained with the pool of human sera. Similar results were obtained in cytometric analysis with another pool of human sera from HIV-1-infected patients and the serum of patient CHA (data not shown). The similarity of the results obtained for the various envelopes with the human sera that, by definition, recognize several epitopes simultaneously and the mAbs that are specific for a single epitope strongly suggests that envelopes from PI have a less cell-surface-specific pattern of expression. These differences in cell surface expression as well as the weak level of mAbs binding to the surface of cells expressing the PI envelope glycoproteins did not allow the antigenic properties of the various recombinant envelopes under their native configuration to be compared.

The low levels of cell surface expression of PI envelope glycoproteins may be due at least in part to the kinetics of viral replication of the various viruses used to clone *env* genes. HIV_{cha} and HIV_{133} PI replicate to only low levels in cultured PBMCs *in vitro.* HIV_{BX08}, the recombinant envelope of which was expressed at the cell surface to a higher level than was observed with the other PI, replicates more efficiently in peripheral blood mononuclear cells (PBMCs). Virus titers were usually from 10^3 to 10^4 infectious units per milliliter (IU/ml) of culture supernatant. The highest levels of expression on the cell surface were observed with TCLA virus envelopes. These viruses have high replicative capacities and titers exceeding 10^5 IU/ml of culture supernatant are usually obtained. Could the transportation of the PI envelope from the cell cytoplasm to the cell membrane be associated with a downregulation of the replication rate of the viruses? This phenomenon can hardly be shown in cells replicating HIV because many factors interfere with virus growth. In the SFV system, the recombinant RNAs are strictly similar except for the sequence of the *env* genes. Consequently, the differences in cell surface expression can only be due to the amino acid sequence of the envelope. All the sequences of the most important signals described as being involved in intracellular trafficking and endocytosis of the HIV envelope glycoproteins were similar in the PI- and TCLA-derived envelope glycoproteins (Berlioz-Torrent *et al.,* 1999). Thus, the signal involved in the differences of cell surface expression between the PI- and TCLA-derived envelope glycoproteins included in this study remains to be identified. However, our current research performed using chimeric envelope glycoproteins (i.e., $gp41_{MN}/gp120_{133}$) indicates that such a retention and/or reinternalization signal is located within the transmembrane envelope glycoprotein (gp41) of the PI (Lebigot *et al.,* manuscript in preparation). In addition, it will be of interest in further studies to determine whether our different recombinant envelope glycoproteins expressed using the SFV system are able to complement infection. We already showed that cells expressing some of these recombinant envelope glycoproteins can fuse and produce syncytia with CD4-positive cells (Verrier *et al.,* 1999).

RIPA and cytometry studies showed that TCLA virus envelopes differed from PI envelopes in antigenicity and in expression levels at the cell surface. The effects on immunogenicity of these specific properties were evaluated in BALB/c mice after immunization with rSFV particles producing the various envelopes individually or with a mixture of rSFV particles producing the various envelopes. Antibody responses were analyzed quantitatively by envelope-specific ELISAs. These ELISAs were performed using cell lysates from electroporated BHK-21 cells as a source of envelope glycoproteins. The envelope glycoproteins in cell lysates were unprocessed and had both gp120 and gp41 epitopes. These recombinant antigens were used in preference to the native envelope glycoproteins derived from virions because the replication rate of HIV_{CHA} and HIV_{133} in cultured PBMCs was too low for production of the minimum amount of protein required for ELISA. The control ELISAs performed with two pools of human sera from HIV-1-infected patients showed that (i) the epitope recognized by the anti-gp120 sheep polyclonal capture antibody D7324 was present and accessible on the various envelopes and (ii) the amounts of envelope glycoprotein captured on the solid phase were similar in all cases. The results of the ELISAs led to three conclusions. First, the immune responses obtained in the groups of mice immunized with the various envelopes were slightly strain-specific. Second, the antibody titers against homologous envelopes were two to three dilutions higher in mice immunized against TCLA virus envelopes than in mice immunized against PI envelopes, showing that PI recombinant glycoproteins are more weakly immunogenic. The weak cell

surface expression of the PI envelope, as shown by cytometric analysis, could have consequences for the intensity of the humoral immune response although cells expressing the envelope *in vivo* probably undergo lysis and release the intracellular envelope. Third, the humoral immune response against the HIV envelope can be broadened by simultaneous immunization with envelope glycoproteins derived from different strains. Similar ELISAs were also performed with the mature gp120 found in the culture supernatants of electroporated BHK-21 cells. The results showed that the weak immunogenicity of PI envelopes was most pronounced if only gp120 epitopes were available on the solid phase (data not shown), which renders difficult the comparison of the results.

The results presented herein suggest that the SFV system produces HIV envelope antigens that have features in common with the native antigens on naturally occurring virions: PI envelopes generally have weaker and more variable antigenicity than TCLA envelopes. Our study also suggests that some PI envelopes may be less expressed on the cell surface than TCLA virus envelopes. Obviously, this phenomenon does not lower the density of envelope glycoproteins present on virion particles since previous reports showed a higher density of envelope glycoproteins present on virions for PI by comparison with TCLA strains (Sullivan *et al.,* 1995; Willey *et al.,* 1994). However, this phenomenon may be indirectly involved, along with the lower accessibility of the neutralizing epitopes on PI envelope glycoproteins, in the escape of HIV-1 PI-infected cells from the immune system *in vivo.*

The weak antigenicity of recombinant PI envelope antigens raises the question whether these PI antigens could induce better neutralizing antibodies than the already tested TCLA envelope immunogens, which have given disappointing results.

MATERIALS AND METHODS

Virus and provirus DNAs

We studied three PI (BX08, CHA, and 133) and two TCLA strains (MN and HXB2) of HIV-1. The coreceptor usage of the PI was determined only for HIV_{BX08}, which uses the CCR5 coreceptor (determination by F. Barré-Sinoussi, Institut Pasteur, Paris; personal communication). For the other two primary viruses, the viral titers obtained after culture *in vitro* with mitogen-activated PBMCs were too low for determination of coreceptor usage. The PI BX08 and CHA were isolated soon after seroconversion (within 6 months). BX08 was provided by H. Fleury and I. Pellegrin (Centre Hospitalier Universitaire Pellegrin, Bordeaux, France). The virus was amplified by one passage in mitogen-stimulated PBMCs from seronegative donors to obtain infected cells for DNA extraction (Moog *et al.,* 1997). Cellular DNA was extracted from cultured PBMC 1 week after infection, using the Wizard genomic DNA purification kit (Promega, Madison, WI). CHA was obtained by coculture of PBMCs from an HIV-infected individual with mitogen-stimulated PBMCs from seronegative donors (Hollinger *et al.,* 1992). Viral growth was assessed by monitoring the release of p24 into the culture supernatant, using a commercial kit (Vironostika HIV-1 antigen, Organon Technika, Oss, The Netherlands). Cellular DNA was extracted from cultured PBMCs harvested after 18 days of culture, using the Wizard genomic DNA purification kit. For the third primary virus (133), cellular DNA was extracted directly from Ficoll-Hypaque-fractionated PBMCs of an infected individual in the acute phase of a primary infection before seroconversion, using the Wizard genomic DNA purification kit. Therefore, the *env* gene of BX08 was cloned from a primary isolate that was passaged several times *in vitro* in PBMCs, the *env* gene of CHA was cloned from proviral DNA as soon as the coculture of PBMCs showed signs of replication (detection of p24 in the supernatant), and the *env* gene of 133 was cloned directly from the uncultured PBMCs of the patient.

For the TCLA $HIV\text{-}1_{MN}$, an aliquot of infectious culture supernatant was incubated with SupT1 cells at 37°C for 1 h. The cells were pelleted and resuspended at a density of 10^6 cells/ml in RPMI containing 10% heat-inactivated fetal calf serum (FCS) and maintained at 37°C under 5% CO_2. Cellular DNA was extracted after 7 days of culture, using the Wizard genomic DNA purification kit. The HXB2 clone was already available in the laboratory (Fisher *et al.,* 1985).

SFV vector construction

PCR amplification of the entire $HIV\text{-}1_{HXB2}$ *env* gene and the construction of the expression vector pSFV-HXB2 have been described elsewhere (Brand *et al.,* 1998). All the other *env* genes were amplified from DNA extracted from the cultured cells or directly from the uncultured PBMCs for patient 133. Nested PCR was required to obtain enough amplification product for further subcloning. Various sets of primers were used depending on the *env* gene to be amplified. The *env* genes from $HIV\text{-}1_{MN}$, $HIV\text{-}1_{BX08}$, and $HIV\text{-}1_{CHA}$ were amplified with the primers (5′-CCAGTAGATCCTAGACTAGAGCCC-3′) and (5′-TGTATTGCTACTTGTGATTGCTCC-3′) for the first round, and (5′-AGGATCCGAAGACAGGCACCATGAGAGTGAAGG-3′) and (5′-GGGATCCATCTTATAGCAAAGCCCTTTCCAA-3′) for the second round. The inner primers were designed to generate a *Bam*HI site at both ends of the amplified fragment. The *env* gene of $HIV\text{-}1_{133}$ was initially amplified using the primers (5′-AGAAAGAGCAGAAGACAGTG-3′) and (5′-ATTGCTACTTGTGATTGCT-3′) for the first round, and (5′-ATTGTCGACTGGCAATGAGAGTGAAGGAG-3′)

and (5′-CAGCCCGGGTTGCCACCCATCTTATAGC-3′) for the second round. *Bam*HI sites were then created by an additional round of PCR amplification using the following primers: (5′-CGGATCCTTGTCGACGCACCATGAGAGTG-AAG-3′) and (5′-GGGATCCATCTTATAGCAAAGCCCTT-TCCAA-3′). All PCR products were inserted into pCRII using the TA cloning system (Invitrogen, Carlsbad, CA). The expression vectors pSFV.HXB2, pSFV.MN, pSFV.BX08, pSFV.CHA, and pSFV.133 were constructed by inserting the corresponding *env* gene fragment into the *Bam*HI site of the pSFV1 vector (Life Technologies, Rockville, MD). All *env* genes inserted into pSFV1 were sequenced and no significant change was detected in the various sequences. All these viruses were of subtype B.

Preparation of recombinant SFV RNA and SFV particles

Recombinant RNA.HXB2, RNA.MN, RNA.BX08, RNA.CHA, and RNA.133 encoding the full-length gp160 glycoproteins were synthesized *in vitro,* using the various expression vectors as templates. For the control, recombinant RNA.β-gal, encoding the β-galactosidase protein, was synthesized from the expression vector, pSFV3 (Life Technologies, Rockville, MD). The recombinant RNAs (rRNAs) were synthesized *in vitro* according to the protocol provided by the manufacturer and the RNA samples were then stored at −80°C.

Recombinant SFV particles were constructed with pSFVhelper2 (kindly provided by P. Liljeström, Karolinska Institute, Stockholm, Sweden). The pSFVhelper2 vector was used as a template for the *in vitro* synthesis of a recombinant RNA derived from the structural genes of SFV. This RNA was used with each of the recombinants, RNA.HXB2, RNA.MN, RNA.BX08, RNA.CHA, RNA.133, and RNA.β-gal, to cotransfect BHK-21 cells, so as to produce recombinant SFV.HXB2, SFV.MN, SFV.BX08, SFV.CHA, SFV.133, and SFV.β-gal particles (for more details, see (Berglund *et al.,* 1993; Brand *et al.,* 1998; Liljeström and Garoff, 1991; Zhou *et al.,* 1994). Recombinant virus stocks were stored at −80°C. The titers of the recombinant virus stocks were determined using Vero cells as described previously (Brand *et al.,* 1998). Titers are expressed as infectious units per milliliter.

Radioimmunoprecipitation assays

BHK-21 cells (10^7 per transfection) were electroporated (350 V, 750 μF) with 5 μg of each rRNA, plated in 75-cm^2 tissue culture flasks, and labeled in cysteine-free medium containing 5% heat-inactivated FCS and 40 μCi of [^{35}S]cysteine per milliliter. The cells were incubated for 16 h at 37°C and lysed with Nonidet-P40 buffer [0.5% Nonidet-P40, 0.5 M NaCl, 10 mM Tris–HCl (pH 7.5)]. Culture supernatants (800 μl/reaction) and cell lysates (80 μl/reaction corresponding to 3×10^5 cells) were immunoprecipitated with a pool of sera from HIV-1-infected individuals (5 μl/reaction) or human mAb F105 (2 μg/reaction; gift from L. Cavacini and M. Posner, Beth Israel Deaconess Medical Center, Boston, MA), recombinant mAb IgG1b12 (2 μg/reaction; gift from D. Burton, The Scripps Research Institute, La Jolla, CA), mAb 447-52D (2 μg/reaction; gift from S. Zolla-Pazner, New York University School of Medicine, New York, NY), or mAb 2F5 (2 μg/reaction, gift from H. Katinger, Institute of Applied Microbiology, University of Agriculture, Vienna, Austria). The F105 and IgG1b12 mAbs react with conformational epitopes overlapping the CD4 binding site of gp120 (Roben *et al.,* 1994; Thali *et al.,* 1991). The 447-52D antibody binds to the V3 determinant of gp120 (Conley *et al.,* 1994). The 2F5 recognizes an epitope located on the ectodomain of gp41 (Muster *et al.,* 1993). For IgG1b12, a secondary anti-human IgG antibody (Fab specific, Sigma, St. Louis, MO) was required. The immunoprecipitated proteins were incubated with protein A-conjugated Sepharose CL4-B beads (Pharmacia, Uppsala, Sweden; 8 mg/reaction) for 18 h at 4°C. They were washed three times with Nonidet-P40 buffer, separated by electrophoresis in 10% SDS–polyacrylamide gels, and detected by autoradiography. Independent experiments, including envelope expression and RIPA with the pool of human sera and the mAbs were repeated at least twice for each envelope.

Immunofluorescence and flow cytometry analyses

Analyses were conducted on BHK-21 cells electroporated with RNA, with or without permeabilization of the cell membrane, and on SFV-infected Vero cells as follows. Nonpermeabilized BHK-21 cells (10^7 per transfection) were electroporated (350 V, 750 μF) with 5 μg of each rRNA and plated in 75-cm^2 tissue culture flasks. The cells were incubated for 16 h at 37°C, harvested, washed once in PBS, pelleted, and resuspended at a density of 10^6 cells per 100 μl in ice-cold PBS containing 2% bovine serum albumin (PBS–BSA). Cell suspensions (100 μl) were incubated for 30 min at 4°C with a pool of sera from HIV-1-infected individuals (0.5 μl/reaction) or with human mAbs F105 (5 μg/reaction), IgG1b12 (2.5 μg/reaction), 447-52D (2.8 μg/reaction), or 2F5 (10 μg/reaction). The concentrations of mAbs used were saturating, as determined in preliminary experiments, except for the 447-52D antibody, which was not purified (culture supernatant) and for which larger amounts were not available. The cells were washed twice in ice-cold PBS–BSA and incubated for 30 min at 4°C with a FITC-labeled anti-human IgG (Fab) conjugate (Cappel/ICN, Costa Mesa, CA) diluted 1:1500 in PBS–BSA. The cells were washed twice in ice-cold PBS–BSA, pelleted, resuspended in 500 μl of ice-cold PBS–BSA, and immediately analyzed by flow cytometry in a Beckon Dickinson FAC-

Star Plus flow cytometer (Beckon Dickinson, San Jose, CA) with Cellquest software. Permeabilized cells were treated with the IntraPrep Permeabilization Reagent (Immunotech, Marseille, France) just before staining and were stained as described above except that all incubations were at room temperature. Cytometry of permeabilized cells was carried out only with the pool of sera from HIV-1-infected individuals.

For cytometric analysis of infected Vero cells, the cells were seeded at 5×10^6 cells per 75-cm^2 culture flask. They were incubated for 12 h and then infected with the various activated rSFV particles at a multiplicity of infection of 5 infectious particles per cell (Brand *et al.,* 1998). The cells were incubated for 16 h at 37°C, treated as described for nonpermeabilized BHK-21 cells, and immediately analyzed by flow cytometry. Envelope glycoprotein antigen recognition was analyzed only with the pool of human sera.

Immunization protocols

Seven different immunization protocols were used, each on a group of six 8-week-old BALB/c mice. In the first (HXB2 mice), second (MN mice), third (BX08 mice), fourth (CHA mice), and fifth protocols (133 mice), animals were inoculated three times with 10^6 IU of SFV.HXB2, SFV.MN, SFV.BX08, SFV.CHA, and SFV.133 particles, respectively. In the sixth protocol (MIX mice), animals were inoculated three times with a mixture (SFV.MIX) containing 0.2×10^6 IU of each of the 5 rSFV particles. In the seventh protocol (β-gal mice), mice were inoculated three times with 10^6 IU of SFV.βgal as a control. Inoculations with SFV particles were performed on days 0, 14, and 36 for each protocol. For each inoculation, SFV particles were activated as previously described (Brand *et al.,* 1998), diluted to 10^7 IU/ml in Dulbecco's phosphate-buffered saline (Sigma), and 100 μl of viral suspension was immediately injected intramuscularly into the thigh of the mouse. Blood samples were collected on days 0 and 50 and mouse sera were stored at −20°C until used for antibody testing.

Envelope-specific enzyme immunoassays

Sera collected from mice treated according to the various protocols were tested by envelope-specific ELISAs for antibodies against the homologous envelope or heterologous envelopes. The envelope-specific ELISA protocol used was derived from the procedure initially described by Moore *et al.* (1994). BHK-21 cells (10^7 per transfection) were electroporated (350 V, 750 μF) with the rRNAs (5 μg per transfection) encoding each of the various envelopes, to provide a source of envelope glycoproteins. BHK-21 cells electroporated with RNA.β-gal were used as a negative control. After electroporation, the cells were plated in 75-cm^2 tissue culture flasks and were incubated for 16 h at 37°C. The culture supernatants were removed. The cells were washed once with PBS and lysed by incubation with 1% Nonidet-P40 in PBS (2 ml/75-cm^2 flask). The cell lysates were centrifuged at 15,000 *g* for 15 min to remove cell debris. The cell lysates were aliquoted and kept frozen at −80°C until use. Luxlon plates (CML, Nemours, France) were coated (100 μl/well) by incubation overnight at 4°C with a 1 μg/ml solution of anti-gp120 sheep polyclonal antibody D7324 (Aalto, Dublin, Ireland) in Tris-buffered saline [TBS; 100 mM Tris-HCl (pH 7.5), 150 mM NaCl]. The plates were washed three times with TBS containing 0.5% Tween 20 (TBS-T). Nonspecific binding sites were saturated by incubation for 1 h at room temperature with 200 μl of 2% newborn calf serum (NBCS) in TBS. Envelope glycoproteins were captured on the solid phase by incubation for 2 h at room temperature with 100 μl of cell lysate (diluted 1:10 in TBS containing 10% NBCS and 1% Nonidet-P40). The plates were washed five times with TBS-T. Sera collected from mice were diluted in TBS containing 0.5% Tween 20, 20% sheep serum, and 10% NBCS (TBS.T.S.N) and 100 μl of each dilution was added to individual wells. A series of 1 in 2 dilutions, from 1:100 to 1:12,800, were analyzed for postimmunization sera collected on day 50, whereas preimmunization sera collected on day 0 were tested only at a dilution of 1:100. The plates were incubated for 1 h at room temperature and washed five times with TBS-T. A goat anti-mouse Ig (Biosource, Camarillo, CA) conjugated with peroxidase and diluted 1:5000 in TBS.T.S.N was added (100 μl/well). The plates were incubated for 30 min at room temperature and washed three times with TBS.T and 100 μl of a mixture of H_2O_2 and *o*-phenylene-diamine was added. The plates were left for 30 min at room temperature in the dark and the color development was then stopped by adding 50 μl of 2 N H_2SO_4. Absorbance at 490 nm was determined.

Antibody titers are expressed as the highest dilution giving an A_{490} value greater than 0.04 for all ELISAs. The cut-off point was determined by testing the preimmunization samples collected from 36 mice at a dilution of 1:100. Each postimmunization sample was tested by ELISA HXB2, ELISA MN, ELISA BX08, ELISA CHA, and ELISA 133 with envelope glycoproteins from cell lysates of BHK-21 cells electroporated with the corresponding rRNA. The mean absorbance with preimmunization sera was 0.011 (SD 0.003). The cut-off value therefore corresponded to the mean absorbance value plus 10 SD.

We determined whether the envelope glycoproteins derived from cell lysates were uncleaved gp160 or mature gp120 by probing with three murine mAbs specific for the gp120 or the gp41. The anti-gp120 antibody was 2F6C12 (gift from N. Piga, BioMérieux). The anti-gp41 antibodies were 13E5F8 (gift from N. Piga, BioMérieux) and mAb 1577 (IntracelI/Neosystem, Strasbourg, France). These mAbs were tested at a concentration of

20 μg/ml in the various ELISA formats. As expected, the cell lysates contained both the gp120 and the gp41 epitopes. We checked whether the amount of envelope glycoprotein captured on the solid phase was similar regardless of the rRNA used. Envelope-specific ELISAs were carried out with two pools of sera from HIV-1-infected patients and two pools of sera from uninfected patients as negative controls (all at 1:100 dilution). A goat anti-human Ig (Biosource) conjugated with peroxidase was used as the secondary antibody.

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