Neutralizing antibodies to the HIV-1 envelope glycoproteins
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Citation for published version (APA):
Labrijn, A. F. (2004). Neutralizing antibodies to the HIV-1 envelope glycoproteins
An antigenic and immunogenic comparison of membrane-associated and soluble recombinant Human Immunodeficiency Virus Type 1 envelope glycoproteins

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Antibody-mediated neutralization of HIV-1 has been shown to correlate with recognition of the mature oligomeric envelope glycoprotein (Env) complex as present on the membranes of virions and infected cells. Although many efforts have been made to mimic the oligomeric nature of the mature Env complex, only few studies have addressed the membrane environment in which the mature Env complex is expressed. In this study we investigated the influence of the membrane environment on both the antigenic and immunogenic properties of Env proteins. For this we isolated membrane-associated and soluble envelope glycoproteins from recombinant vaccinia virus infected cells expressing the env gene of a prototypic R5 HIV-1 clone (IHI), by using the mild non-ionic detergent n-octyl β-D-glucopyranoside (OG) in combination with size-exclusion- and affinity chromatography. Screening of a number of human HIV-1 seropositive sera for binding to Env in membrane-associated and soluble forms indicated that the majority (up to ~97%) of the antibodies elicited in the course of natural infection recognized epitopes that are not available on the membrane-associated form. Sequential serum samples from one patient showed that samples obtained during established infection preferentially recognized soluble Env forms. Interestingly, samples obtained shortly after seroconversion, however, preferentially recognized membrane-associated Env forms. Immunization of rabbits with both Env preparations revealed that membrane-associated and non-membrane-associated Env elicit qualitatively different responses. Overall these findings indicate that the membrane environment markedly changes the antigenic and immunogenic properties of the Env complex, which may have important conceptual implications for development of vaccines able to induce neutralizing antibodies.

### Introduction

The human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein (Env) complex has been identified as the primary target for neutralizing antibodies (22, 41, 45). The Env complex is synthesized as a precursor glycoprotein, gp160, which is assembled as a trimer, (gp160)₃. The gp160 is subsequently cleaved into the gp120 surface subunit and the gp41 transmembrane subunit, resulting in the mature oligomeric Env complex (gp120-gp41), on the surface of infected cells and, eventually, on virions.

Early studies identified recombinant monomeric gp120 and gp160 subunits as potential vaccine candidates. Monomeric Env, however, elicits poor neutralizing responses to primary isolates of HIV-1 in humans (7, 18, 27, 35, 48, 50). Indeed, experiments in vitro have shown that most potent neutralizing antibodies appear to be distinct, not in terms of recognition of monomeric gp120 or gp160, but in term of recognition of the mature oligomeric Env complex (6, 15, 34, 40, 43, 44). Hence, emphasis regarding potential vaccine candidates for HIV-1 infection has shifted to the mature trimeric Env complex.

In an effort to better mimic the mature oligomeric complex, soluble oligomeric gp160 (47) and gp140 constructs (gp120+gp41 ectodomain construct lacking the cleavage site) have been produced (3, 9-11). However, the constructs made thus far could not elicit an antibody response that could neutralize heterologous primary isolates (9, 33, 39, 47). Furthermore, a study using SIV derived gp140 showed impaired co-receptor interactions (12). Taken together these data suggest that the soluble oligomeric Env tested so far does not accurately mimic native oligomeric Env, as expressed on infected cells or virions.

One obvious difference with the native oligomer is that the gp140 oligomers are uncleaved and hence may not expose all epitopes accessible on native Env. Due to the lability of the non-covalent interaction between gp120 and gp41 (17, 28, 29, 37), the processed oligomer is difficult to isolate. A disulfide-bond stabilized variant of gp140, which still contains the gp120/gp41 cleavage site, has been constructed and may better mimic native Env (1). Another factor that could account for the difference in immunogenicity of the soluble oligomers is the absence of a membrane environment. Antigenic surfaces that are occluded by the virion membrane (23, 51), are exposed on soluble forms, which could skew the antibody response towards specificities that are not accessible on the virion and thus non-neutralizing (e.g. (10)).

The aim of the present study was to compare the antibody response to Env complexes in a membrane context with the response to soluble gp120/gp160. To this end we constructed a recombinant vaccinia virus (rVV) expressing the env gene of a prototypic R5 HIV-1 clone (IHI) under control of a vaccinia late promoter (p11). We used the mild non-ionic detergent n-octyl β-D-glucopyranoside (OG) in combination with size-exclusion- and affinity chromatography to isolate membrane-associated and soluble Env from infected cells (20). Human seropositive serum reactivity to the Env preparations was compared. Additionally, rabbits were immunized with the Env preparations and the immune sera analyzed.

### Materials & Methods

#### Cells, viruses and recombinant proteins

Monocytes, more than 95% pure, were isolated from an HIV-1 antibody negative donor essentially as described by Figdor et al. (13). Briefly, peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood by Percoll density gradient centrifugation. Monocytes were enriched by elutriation. To obtain macrophages, the monocytes were cultured for five days (37°C, 5% CO₂) at a concentration of 10⁵ cells/ml in endotoxin-free Iscove's modified Dulbecco's medium (IMDM; Biowhittaker, Verviers, Belgium) supplemented with 10% (v/v) pooled human serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). CV-1 cells (African green monkey kidney cell-line; ATCC CCL70), RK-13 cells (rabbit kidney fibroblast cell-line) and HeLa cells (human cervical carcinoma cell-line) were obtained through the American Type Culture Collection (ATCC, Manassas, VA) and cultured in IMDM, supplemented with 10% (v/v) fetal calf serum (FCS; Biowhittaker), penicillin (100 U/ml) and streptomycin (100 µg/ml)
Antisera and monoclonal antibodies

The HIV-1 V3 loop-specific mouse monoclonal antibodies (mAbs) IIIB-Antisera and monoclonal antibodies obtained from the NARRRP (contributed by Immunodiagnostics, Woburn, MA) were produced by stably transfected CHO cells and were obtained from the NARRRP (contributed by Immunodiagnostics, Woburn, MA).

PCR amplification of envelope sequence

MDMs were infected with 10^4-10^5% tissue culture infective doses (TCID_{50}) per ml of virus stock. HIV-1 infected MDMs were used. Subsequently, viral DNA was amplified with primers TATGGAGCGCGGCTTGGTCAGCTTGATAGAAGAGAGGCAGGACAGACAGACGGCA, which were designed to amplify a region of 888 bp of the envelope, covering the last part of gp41 (positions 7148-7172) was used to amplify a 965 bp. A unique restriction site (underlined) and positions of the primer in the HIV-1 clone genome are indicated in parentheses. Primer-set A2 5'-TAT CTA GAG CAG AAG AGC AT-3' and the stopcodon Optimal PCR conditions were 1 x 5' 95°C, 30 cycles 7942-7971) was used to amplify a region of 824 bp, covering the second site of p11-ATA18. resulting in plHI.

CHAPTER 4

Construction of plasmid transfer vector

The pGEM-T vectors containing the PCR fragments were digested with the proper restriction enzymes in accordance with the instructions of the manufacturer (Gibco BRL). Reconstitution of the full-length env gene, designated IHI, was done by subsequently ligating the three fragments together in the pSP72 vector (Promega, Madison, WI). The resulting construct was termed p160B-ali. Fragment borders were checked by sequencing and restriction enzyme digestion. To insert the env gene into the vaccinia virus genome, the complete gene was first inserted into plasmid transfer vector p11-AT1A18; downstream from the late vaccine promoter (p11) and flanked by vaccinia sequences encoding the viral thymidine kinase (TK). For this the p160B-ali was digested with Xbal and EcoRV, blunt-ended with Klenow polymerase and cloned into the Smal site of p11-AT1A18, resulting in pHI.

Transfection and isolation of recombinant viruses

To generate the recombinant viruses the general procedure as described by Macak et al. was used (26). In short, HuTK- cells were infected with wild-type (TK+) vaccinia strain WR. At 3 hours post infection (p.i.) calcium phosphate precipitated plasmid DNA (pH1) was added to allow homologous recombination. Cells were harvested 48 hours p.i. and TK recombinants were selected from infected cell lysates by a plaque assay on HuTK- cells in the presence of 100 μg/ml 5-bromo-oxyuridine (BUDR). (Sigma). Independent plaques were plaque-purified and the recombinant vaccinia viruses (RVV) was grown to large stocks in CV-1, RK-13 or HeLa cells.

DNA sequence analysis

Nudeic acid sequencing was carried out using an ABI Prism 377 automated DNA sequencer (Perkin-Elmer, Norwalk, CT) with a Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA).

Metabolic labeling of adherent cells

Pulse chase experiments were done as described before by Braakman et al. (2). Subconfluent cells expressing gp160120 were washed once with Hank's balanced salt solution (HBSS; Gibco-BRL) and preincubated for 2 hours in methionine and cysteine free starvation medium. At time zero, the media was removed and cells were pulse labeled for 10 minutes with 50 μCi of in vitro [%S]-labeling mix (Amersham Pharmacia Biotech, Uppsala, Sweden) and chased for various times. The chase was started by the addition of complete medium containing excess unlabeled cysteine and methionine. Cytochrome was included in the chase to stop elongation of nascent chains. The chase was stopped by transferring the cells to ice and washing them with ice-cold HBBS containing 20 mM iodocetamide to alkylate free sulfhydryl groups in the labeled proteins. The cells were lysed in 0.5% (w/v) Triton X-100 in MNT (20 mM MES, 100 mM NaCl, 30 mM Tris-HCl, pH 7.4) containing 20 mM iodocetamide and protease inhibitors (chymostatin, leupeptin, antipain, pepstatin, PMSF and EDTA). Lysates were used directly for immunoprecipitation.

Immunoprecipitation

Protein A Sepharose 4B fast flow beads (Amersham Pharmacia Biotech) were incubated for 30 minutes at 4°C with polyclonal rabbit antisemum CLB-40336. 1 ml immunoprecipitation 100 μl of lysate was added and incubated for 15 hrs at 4°C. The immunoprecipitates were washed twice with buffer containing 0.05% (w/v) Triton X-100, 0.05% SDS and 300 mM NaCl in 10 mM Tris-HCL, pH 8 for 5 minutes at RT. The washed pellets were resuspended in 10 μl 10 mM Tris-HCL, pH 6.8 after which non-reducing sample buffer was added to a final concentration of 200 mM Tris-HCL, pH 6.8, 3% SDS, 10% glycerol, 0.004% bromophenol blue and 1 mM EDTA. After heating for 5 minutes at 95°C, samples were subjected to non-reducing SDS-PAGE (7.5%).

Solubilization of Env

Membrane-associated Env. Membrane-associated Env was isolated as described previously (1986). In short, HeLa or RK13 cells infected with RVV (48-72 hrs p.i.) were harvested and subsequently incubated for 30 minutes on ice in lysis buffer (PBS supplemented 200 μM PMSF and 80 μM Pepstatin) containing 1% (w/v) n-octyl-(1-D-glucopyranoside (OG, Sigma, St.Louis, USA). After removal of cell-debris (10 min, 20000g, 4°C), the lysate was fractionated using an AGA ultragel-34 gel-filtration column (Bioapra, Cergy-Saint-Christophe, France), equilibrated in the BRL, Rockville, MD) and checked by DNA sequence analysis.
same buffer except for an OG concentration of 0.05 % (w/v), which is below the critical micelle concentration (CMC). Fractions (500μl) were collected, and the turbidity (vesicle formation) was assayed by measuring A590nm, as well as the Env content by means of GNA-capture ELISA (see below).

NP-40 treatment: To liberate the Env proteins from the membrane-context in the GNA-capture ELISA, the Env preparations were diluted in PBS/ 0.4% ELK/ 0.05% gelatine/ 0.5% Nonidet P-40 (NP-40) in the antigen incubation step.

Purified Env: Soluble-Env containing fractions, within the fractionation range of the column, were pooled and applied onto a 2 ml bed of Lentil-Lectin-Sepharose beads (Amersham Pharmacia Biotech), layered on top of a 25 ml gel-filtration column of G25-fine (Pharmacia), pre-equilibrated with the same buffer as used for the ACA-34 column. The column was washed with the same buffer until no protein was detected. The glycosylated proteins were then eluted from the column by competition with 0.5 M Methyl-α-D-Manopyranoside (Sigma) in PBS. Fractions were monitored for Env content with an ELISA (see below). Env-containing fractions were pooled, and, if necessary, concentrated on a Centricon-10 filter (Amicon, Beverly, MA). The purity of the envelope proteins was checked by 8% SDS-PAGE in the presence of 8 M Urea.

Enzyme-linked immunosorbent assay (ELISA)

GNA-capture ELISA: The wells of Falcon MicroTest III 96-well plates (Becton Dickinson, Franklin Lakes, NJ) were coated with 25 μl of PBS containing 20 μg/ml of Galanthus nivalis agglutinin (GNA; Boehringer Mannheim, Mannheim, Germany) per ml O/N at RT. Plates were blocked with PBS containing 4% (w/v) non-fat dry milk (Campina, Eindhoven, The Netherlands) and 0.5% (w/v) gelatine (Merek; Darmstad, Germany) for 1 hr at RT and washed with PBS containing 0.02% (w/v) Tween-20.

Antigen preparations were added to the wells for 1 hr at 37°C and the captured recombinant Env was then incubated with mono- or polyclonal antibodies for 1 hr at 37°C. The bound antibodies were detected with either alkaline-phosphatase-conjugated (AP) goat anti-human F(ab); immunoglobulin G (IgG) (Pierce, Rockford, IL) or AP-conjugated goat anti-rabbit IgG (Pierce) and β-Nitrophenyl Phosphate substrate (Sigma). Peptide ELISA: Peptide ELISA was carried out as described previously by Huisman et al. (21).

Results

Cloning and expression of the HIV-1 env gene

The env gene of a prototypic R5 HIV-1 isolate ACH172.B-al was PCR amplified and cloned downstream of a p11 late promoter in vaccinia expression vector p11-ATA18. The resulting construct, pHl, was checked by DNA sequence analysis and recombinant vaccinia viruses, rVVi, were grown. To determine intracellular synthesis and maturation of EnvVi, rVVi infected HeLa cells were pulse-labeled with [35S]-methionine and chased for 0, 5, 15, 30, 60, 120, 240 and 480 minutes and O/N. Lysed cells (A) and culture supernatant (B) were immuno-precipitated with rabbit polyclonal antiserum CLB-W61D to HIV-1 Env and analyzed by SDS-PAGE.

Figure 4.1: Intracellular synthesis and processing of the HIV-1, envelope gene products. HeLa cells were infected with rVVim, at 5 hrs p.i. the cells were pulse-labeled with [35S]-methionine and chased for 0, 5, 15, 30, 60, 120, 240 and 480 minutes and O/N. Lysed cells (A) and culture supernatant (B) were immuno-precipitated with rabbit polyclonal antiserum CLB-W61D to HIV-1 Env and analyzed by SDS-PAGE.

the gp41 moiety.

Isolation of membrane associated and soluble envelope glycoproteins

Non-ionic detergents are inefficient in dissociating non-covalent protein-protein interactions. This property has made them a widely used tool to solubilize and isolate viral envelope proteins (20, 36). The non-ionic detergent n-octyl β-D-glucopyranoside (OG) differs from most other non-ionic detergents typically used in biochemistry in having a very high critical micelle concentration (CMC). This property, in combination with size-exclusion chromatography, was used to capture membrane-associated Env from infected RK13 (rabbit) or HeLa (human) cells in detergent-lipid vesicles as described previously (Chapter 3). The EnvVi-containing-vesicles were collected in the fractions representing the void volume of the gel-filtration column and will further be referred to as OG-P1i. The remaining fractions containing EnvVi, falling within the fractionation range of the column, were collected, purified on a Lentil-Lectin-Sepharose column and further referred to as LLSi. Both OG-P1i and LLSi were subjected to western blot analysis with anti-HIV-1 Env antiserum from rabbits. Similar to the results shown previously (Chapter 3; Fig. 3.1A insert), both preparations contained gp160 and gp120 species (data not shown). The predominance of uncleaved Env in OG-P1i is likely the result of saturation of the cellular protease responsible for Env cleavage due to the high levels of protein associated with vaccinia driven expression.

Influence of the membrane environment on the antigenicity of EnvVi

To determine the influence of the membrane context on Ab recognition, HeLa cell-derived OG-vesicles were disrupted by incubation with 0.5% (w/v) Nonidet P-40 (NP), a more stringent detergent, and further referred to as NP-P1i. A panel of sera from asymptomatic HIV-1 infected individuals at different stages of disease progression were tested for binding to both preparations. Half maximal binding concentrations (EC50) were determined and compared. NP-treatment of OG-P1i increased the binding of the human sera to the membrane-associated Env (Fig. 4.2A). This is presumably
caused by increased availability of the epitopes that are not accessible in the membrane-context. The increase in binding ranged from approximately 2- to 30-fold was observed, suggesting that approximately 50-97% of the antibody response may be directed to epitopes that are not accessible in the membrane-context. Alternatively the increase in binding could be the result of better binding of the liberated Env to the GNA, used to capture the Env. However, the fact that the vaccinia specific serum binds only 1.25-fold better to NP-P1\textsubscript{111} does not support this.

Next, sequential serum samples, drawn shortly after seroconversion (SC), were obtained from an HIV-1 infected individual, designated patient W (42). Analysis of serum-binding to both OG-P1\textsubscript{111} and NP-P1\textsubscript{111} revealed a switch in the preferential recognition of both Env preparations. Whereas serum samples drawn before 7 weeks post-SC preferentially recognized membrane-associated Env, serum samples drawn after 7 weeks post-SC displayed a preference for NP-treated Env (Fig. 4.2B). The preference for NP-treated Env increased with time. From three of the asymptomatic HIV-1 infected individuals tested in Fig. 4.2A, serum samples drawn at earlier time-points were available (drawn between 8 weeks and 7 months of suspected SC). In agreement with the observations above, these samples also showed stronger binding to NP-treated Env compared to membrane-associated Env and this preference in binding increased as the serum was taken at later time-points (data not shown).

**Immunogenicity of Env\textsubscript{111} preparations**

To determine the influence of the membrane environment on the immunogenicity of Env\textsubscript{111} preparations, OG-P1\textsubscript{111} and LLS\textsubscript{111}, produced from rVV\textsubscript{111} infected RK13 cells, were injected into rabbits. The immune and pre-immune sera were used to monitor the fractions of a gel-filtration of a 1% (w/v) OG-lysate of rVV\textsubscript{111} infected HeLa cells by means of ELISA (Fig. 4.3A). Antibody titers from the OG-P1\textsubscript{111} immunized animals were lower compared to the LLS\textsubscript{111} immunized animals. To obtain a comparable signal, serum derived after the third immunization with LLS\textsubscript{111} (CLB-17-3) was diluted approximately one log more compared with serum derived after the fourth immunization with OG-P1\textsubscript{111} (CLB-11-4). Both sera, CLB-11-4 and CLB-17-3, preferentially recognized the peak from which their respective immunogens were derived, i.e. OG-P1\textsubscript{111} and LLS\textsubscript{111} respectively.

As it is likely that OG-P1\textsubscript{111} also contains vaccinia-derived membrane-associated proteins, the contribution of vaccinia specific antibodies in the recognition of OG-P1\textsubscript{111} by CLB-11-4 was determined. This was done by comparing the binding of CLB-11-4 to OG-P1\textsubscript{111} with the binding to a similar preparation derived from HeLa cells infected with a rVV encoding T7 RNA polymerase (OG-P1\textsubscript{T7}). Both antigen preparations were tested in an ELISA that was normalized for vaccinia protein content with a vaccinia specific polyclonal rabbit antiserum (Fig. 4.3B). The recognition of OG-P1\textsubscript{111} was 5-
to 10-fold higher compared to the recognition of OG-P1$_{17}$, indicating that approximately 10-20% the OG-P1$_{17}$ specific antibodies are directed to vaccinia proteins.

To further define the specificity of the sera, the binding ability to several Env related antigens was assessed by means of ELISA and compared with other Env specific rabbit sera (for each antigen one representative result from three experiments is shown in Fig. 4.4A+B). A relative rank order was determined for several antigens: OG-P1$_{17}$ (CLB-11-4 > CLB-17-3 > CLB-W61D = CLB-40336), NP-P1$_{17}$ (see below; CLB-11-4 = CLB-17-3 > CLB-W61D = CLB-40336), LLSIHI (CLB-17-3 = CLB-W61D > CLB-40336 > CLB-11-4), gp120$_{W61D}$ (CLB-17-3 > CLB-W61D > CLB-40336 > CLB-11-4) and gp120$_{40336}$ (CLB-17-3 = CLB-W61D > CLB-40336 > CLB-11-4). Overall, CLB-11-4 was the highest-ranking serum against the membrane-associated Env (OG-P1$_{17}$), even when taken into account that 10-20% of the signal is caused by vaccinia specific antibodies. The CLB-17-3 serum was among the highest-ranking sera against all soluble Env forms (NP-P1$_{17}$, LLSIHI, gp120$_{W61D}$ and gp120$_{40336}$).

Subsequently, the immune-sera were tested in a PBMC-based neutralization assay. No significant neutralizing activity could be observed at a serum dilution of 1:16 (data not shown).

**Discussion**

A recombinant HIV-1 Env complex that mimics the antigenic properties of the native Env as present on virions could play an important role in HIV-1 vaccines and in the research addressing antibody mediated HIV-1 neutralization. As a consequence many efforts have been made to make soluble oligomeric constructs (3, 9-11). However, the constructs made thus far could not elicit an antibody response that could neutralize a broad range of primary isolates (9, 33, 39, 47). Our interest was directed at the influence of a membrane environment on the antigenic and immunogenic properties of Env proteins and the use of cell-membrane expression for the isolation of oligomeric Env.

For this purpose we constructed a recombinant vaccinia virus expressing the Env of a prototypic R5 HIV-1 virus and infected HeLa (human) and RK13 (rabbit) cells. To isolate the membrane-associated Env$_{W61D}$ we used the mild non-ionic detergent n-octyl β-D-glucopyranoside (OG) in combination with size-exclusion chromatography. This detergent has been widely used to solubilize viral membrane glycoproteins without dissociating non-covalent interactions between protein subunits (20, 36). Due to its characteristic high critical micellar concentration (CMC) it is relative easy to reconstitute the membrane by lowering the OG concentration. In this study HIV-1$_{W61D}$ Env expressing cells were solubilized with 1% (w/v) OG and separated on a gel-filtration column. The column was equilibrated in 0.05% (w/v) OG, which is below the CMC. In this way membrane reconstitution occurs during passage over the column and membrane associated proteins are captured in detergent-lipid vesicles, designated OG-P1$_{W61D}$ (Chapter 3).

Screening of a number of human HIV-1 seropositive sera for binding to Env$_{W61D}$ in membrane-associated (OG-P1$_{W61D}$) and non-membrane-associated (NP-P1$_{W61D}$) forms, indicated that the majority (~50-97%) of the antibodies elicited during natural infection recognized epitopes that are not available in the membrane-associated form. In principle, this is in agreement with the hypothesis proposed by Parren and Burton (5, 32), that the majority of human Ab response to HIV-1 is induced by ‘viral debris’, e.g., uncleaved gp160 or disassembled envelope. However, whereas the authors contribute this to the phenomenon of ‘original antigenic sin’, the data obtained with the sequential serum samples obtained shortly after seroconversion presented here and in a previous publication (25) argue against this. We demonstrate that the antibody response against the ‘original antigen’ recognizes membrane-associated Env species and that this response evolves towards recognition of soluble Env species (or membrane-associated Env species that share antigenic determinant with soluble Env species). So more likely the antibody response against the ‘original’ functional Env is skewed towards a response to non-functional Env as a result of antigenic competition caused by an excess of viral debris after established infection. This process occurs very quickly as strong recognition of the soluble Env becomes apparent within weeks post seroconversion.

To compare antibody responses elicited by membrane-associated and soluble Env, we immunized...
rabbits with these envelope species. Although derived from the same env gene, both OG-P1
and LLSI
preparations elicited qualitatively different Ab responses, as illustrated by the difference in rank-order to the non-
membrane associated Envi
preparations (NP-P1
and LLS).
For example, CLB-11-4, which is among high-
ranking binders to NP-P1
, is among the lowest-ranking binders to LLS and monomeric recombinant envelope species. Additionally, CLB-W61D and CLB-40336, which were elicited by immunizing with gp160 of HIV-1 strains W61D and IIIB respectively, also reacted more strongly with soluble Env species.

During Env synthesis, the gp160 glycoprotein precursor undergoes several maturation steps including assembly into a trimer, modification of the carbohydrate side chains and cleavage by a cellular furin-type proteases (19). The efficiency of this pathway is limited, as only a small part gets cleaved into the mature Env complex, (gp120-gp41) (8, 31, 49). The majority of gp160 produced is delivered to a lysosomal compartment and degraded (4, 49). Considering that our isolation method was based on capturing the membrane-associated Envi
 (OG-P1I), the soluble Envi
species represented in LLS would then, for the majority, constitute the gp160 species that are recycled via the cytosol and targeted to the lysosome, and the gp120 proteins that are shed from the membrane due to the experimental manipulations.

Preliminary analysis of resistance to Endoglycosidase H (Endo H) treatment suggested that LLSI
indeed contained relatively more Endo H sensitive Env species, which is indicative of more immature Env species (data not shown). The OG-P1I contained more Endo H resistant species (data not shown). The presence of Endo H resistant gp160 in OG-P1I agrees with the finding that cleavage is not a prerequisite for surface targeting, as has been demonstrated for HIV-1 infected cells (30).

The presence of the membrane-associated uncleaved Envi
could explain the inability of OG-P1I to elicit a neutralizing Ab response. This is further illustrated by the fact that OG-P1I could not select for a broad-neutralizing Fab, b12, from a phage display library (Chapter 3). As the uncleaved oligomer exposes several epitopes that are occluded in the cleaved oligomer (10), the observed effect of the membrane environment is likely an underestimation of the situation in vivo. Alternatively, with the recent demonstration that Env spikes on virions are heterogeneous (38), the presence of non-functional spikes in the OG-P1I preparation could have contributed to its inability to elicit neutralizing Abs. Finally, the inability to elicit neutralizing antibodies could be the result of sub-optimal immunization conditions. The water-in-oil emulsion side chains used in this study have been described to influence antigen conformation (46), thus skewing the antibody response to the non-functional epitopes.

The fact that all sera obtained from injection with monomeric Env, irrespective of Env origin, are directed to the non-membrane associated epitopes to a similar degree, implies a major contribution of antibodies to common epitopes. As only antibodies capable of binding to the epitopes exposed on the limited surface of mature oligomeric Env can effectively inhibit HIV-1 infection (34), one could argue that these are the only epitopes that experience an antibody-mediated selective pressure. The other surfaces, not accessible on the mature oligomeric Env, thus could expose common motifs that are the result of other selective pressures, which display common immunogenic epitopes recognized by the sera. The cross-reactive antibodies are thus mainly directed to the non-membrane-associated epitopes. This is in accordance with the observation that the immune serum from the rabbit immunized with membrane-associated Env, poorly recognized the soluble Env of the other strains.

Overall these data suggest that the membrane environment markedly changes the antigenic and immunogenic properties of Env. We suggest that to direct the immune response to the relevant epitopes this should be taken into account. Furthermore, infected cells as a source of membrane-associated Env and a combination of solubilization with a mild detergent (OG) and gel-filtration presents a relative easy way to isolate membrane-associated Env from Env expressing cells. Improvement of both the immunogenicity and the relative content of processed Env over unprocessed Env would be desirable and would warrant further investigations.

Acknowledgement

We are grateful to Dr. H.M. Weigel for providing patient materials.

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