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
Novel strategy for the selection of human recombinant Fab fragments to membrane proteins from a phage-display library

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Traditionally, the selection of phage-display libraries is performed on purified antigens (Ags), immobilized to a solid substrate. However, this approach may not be applicable for some Ags, such as membrane proteins, which for structural integrity strongly rely on their native environment. Here we describe an approach for the selection of phage-libraries against membrane proteins. The envelope glycoproteins (Env) of the Human Immunodeficiency Virus type-1 (HIV-1) were used as a model for a type-1 integral membrane protein. HIV-1 Env, expressed on the surface of Rabbit Kidney cells (RK13) with a recombinant vaccinia virus (rVV), was solubilized using the non-ionic detergent n-Octyl β-D-glucopyranoside (OG). Membrane associated Env was reconstituted into vesicles by the simultaneous removal of detergent and free monomeric Env subunits by gel-filtration. The resulting antigen preparation, termed OG-P1I1, was captured on microtiter plates coated with Galanthus nivalis agglutinin (GNA) and used for rounds of selection (panning) of a well-characterized phage-display library derived from an HIV-1 seropositive donor. Simultaneously an identical experiment was performed with OG-P1I1 vesicles disrupted by Nonidet P-40 (NP-P1I1). Both membrane-associated and soluble Ags selected for vaccinia-specific clones (OG-P1I1: 59/75 and NP-P1I1: 1/75), and HIV-1-specific clones (OG-P1I1: 11/75 and NP-P1I1: 65/75) using our approach. Hence, the novel panning strategy described here may be applicable for selection of phage-libraries against membrane proteins.

Introduction

The display of antibody fragments on the surface of filamentous phages and the subsequent selection of antibodies (Abs) have proven an effective tool for the isolation of Abs to defined Ags (7, 25, 51). Most traditional selection strategies depend on purified antigens immobilized to a solid-phase matrix. However, some antigens may not be available in purified form, either because their identity is unknown or because the purification procedure influences the conformation, which in turn may affect the selected antibody repertoire. For example, integral membrane proteins are often dependent on their native environment for their physiological conformation, which makes them difficult to purify. As a result, alternative selection strategies based on whole cells (10, 12, 22, 32, 34, 46), tissue sections (49) or even selection in vivo (27) have been developed. Due to the great abundance of irrelevant antigenic moieties on cell-surfaces, the preferential selection of specific Abs with their physiological conformation strongly rely on their native environment. Here we describe an approach for the selection of phage-display libraries against membrane proteins.

To solubilize membrane proteins while retaining their physiological conformation, mild non-ionic detergents are often used. One such detergent, n-Octyl β-D-glucopyranoside (OG), has been used in the solubilization and reconstitution of viral envelope proteins of Semliki Forest virus (SFV) (23), vesicular stomatitis virus (VSV) (15, 38), influenza virus (26, 48) and Sendai virus (1, 20, 45). The characteristic property of OG is that it has a high critical micellar concentration (cmc) making removal of the detergent very easy.

In this study, we describe a strategy for the solubilization of membrane proteins with OG and reconstitution by gel-filtration. HIV-1 Env was used here as a model system for a type-1 integral membrane protein. The obtained Ag preparations were assessed for their ability to select HIV-1 Env specific clones from a well-characterized phage display library obtained from the bone marrow of an HIV-1 seropositive donor. We here show that our approach results in the isolation of novel Abs against Env.

Materials & Methods

Antibodies and purified proteins. The following antibodies (Abs) were used in this study: rabbit antiserum against gp160/gp120 of HIV-1m Env, produced in CHO cells (CLB-W61D, produced in our laboratory), mouse ID-120-17 monoclonal (mAb), directed to a conformational epitope in the C1 domain of HIV-1 gp120 (unpublished data), horse/rabbit polyclonal (HRP)-labeled, mouse anti-M13 mAb (Amersham Pharmacia Biotech, Upsalla, Sweden), human IgG1b12 and phage b12, directed to an epitope overlapping the CD4 binding site of gp120 were kind gifts from Dr. P. Parren (The Scripps Research Institute, La Jolla, CA), rabbit anti-vaccinia virus (VV) polyclonal (p)Ab (Biotrend, Köln, Germany) and HRP-labeled goat-antibody IgG (Pierce, Rockford, IL). Biotinylation of IgG1b12 was done by incubation with Sulfo-NHS-LC-biotin (Pierce) according to manufacturer's instructions. The following purified proteins were obtained through the NIH AIDS research and reference reagent program (NARRRP); HIV-1 gp120m, and gp120m (contributed by Immunodiagnostics, Woburn, MA), soluble (s)CD4 (contributed by Dr. R. Sweet). C. inhibitor was obtained from the CLB (Amsterdam, The Netherlands).

Viruses and Cell lines. RK13 and HeLa cell cultures were cultured in Iscove's modified Dulbecco's medium (IMDM; Biowhittaker, Verviers, Belgium) supplemented with 10% (v/v) fetal calf serum (FCS; Biowhittaker), penicillin (100 U/ml) and streptomycin (100 ug/ml) at 37°C in 5% CO2. A recombinant vaccinia virus (rVV) expressing the HIV-1 Env gene (rVV1Env) derived from the ACH172.B-al virus isolate (16), was constructed as described (Chapter 4).

Antigen preparations. Confluent RK13 or HeLa cell monolayers, grown in 75 cm2 flasks, were infected with 5 MOI rVV1Env. After virus adsorption in 5 ml of culture medium for 1 hour at 37°C, fresh culture medium was added and the cells were further incubated at 37°C. After 48-72 hours of infection the cells were harvested and washed with phosphate buffered saline (PBS) at pH 7.4. Subsequently, the cells were incubated for 30 minutes on ice in 0.5% (v/v) n-Octyl β-D-glucopyranoside (OG, Sigma, Sigma, Sigma).
(70 µg/ml) was added. After overnight growth at 30°C, the cells were harvested by centrifugation (30 min, 14,000 x g, 4°C) and the supernatant discarded. The pellet was resuspended in 1 ml of PBS/1% (w/v) BSA and centrifuged (5 min, 14,000 x g). The supernatant was used for the next round of panning. After four rounds of panning individual colonies were grown and the phages were prepared as described above for screening in GNA-capture ELISA (see below).

**Westernblot analysis.** The fractions of interest were diluted in sample buffer containing SDS and DTT and heated to 95°C for 5 minutes. Samples were analyzed by SDS-PAGE (4-12%, Novex, San Diego, CA) under reducing conditions. Subsequently, proteins were transferred to nitrocellulose membranes and blocked according to the manufacturer's instructions. For the detection of HIV-1 Env, polyclonal rabbit serum CLB-W61D was used. The bound antibodies were detected with HRP-labeled goat-anti rabbit IgG in combination with ECL plus Western blotting detection system (Amerham Pharmacia Biotech).

**Flow cytometry.** All reagents were centrifuged (45 min, 38,000 x g, 4°C) before use to remove aggregates. Vesicles were diluted 1:5 in ice-cold 10 mM HEPES/150 mM NaCl/5 mM KCl/2 mM CaCl2/2 mM MgCl2/0.1% (w/v) BSA/0.02% (w/v) NaN3 at pH 7.2 (FACS buffer) and incubated with 1 uM of biotinylated mAb IgG1b12 for 30 min at 37°C. For scFv binding, the vesicles were incubated with 25 or 5 uM of scFv before addition of IgG1b12. The vesicles were washed once, by dilution 1:15 in FACS buffer followed by centrifugation (45 min, 38,000 x g, RT). Next, the vesicles were simultaneously incubated with fluorescein-isothiocyanate-labeled Annexin V (AnnV-FITC; Bender MedSystems, Vienna, Austria) diluted 1:10 in FACS buffer and phycoerythrin-labeled Streptavidin (Strep-PE; Becton Dickinson, San Jose, CA) diluted 1:25 in FACS buffer. The vesicles were then analyzed with a FACSCellibur flow cytometer with CellQuest software (Becton Dickinson). A total of 30,000 events were measured per sample.

Panning of the antibody phosphate display library. A Feb display library, comprised of 200,000 filamentous phage derived from the bone marrow of an HIV-1 seropositive donor as described previously (8, 39), was kindly provided by Dr. D. Burton (The Scripps Research Institute, La Jolla, CA). For each round of panning, the wells of Falcon MicroTest III 96-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) were coated overnight at RT with 25 µl of Galanthus nivalis agglutinin (GNA; Boehringer Mannheim; 20 µg/ml). Plates were blocked with PBS containing 4% (w/v) non-fat dry milk (NFDM; Campina, Eindhoven, The Netherlands) and 0.5% (w/v) gelatine (Merck, Darmstadt, Germany) for 1 hr at RT and washed with PBS. OG-P1h was added to the plates, diluted 1:5 in 0.4% non-fat dry milk/0.05% (w/v) gelatine in PBS supplemented with either 0.05% OG (column concentration) or 0.5% Nonidet P-40 (NP-P1h) and incubated for 1 hr at 37°C. For round 100 µl of phage suspension was incubated with antigen for 2 hrs at 37°C. Unbound phages were removed by washing 10 times with PBS/0.02% Tween-20 over a 1 hr period. The bound phages were eluted with 100 µl of 0.1 M glycine buffer (pH 2.2), and neutralized with 6 µl of 2 M Tris base. The eluted phages were used to infect 2 ml of freshly grown E. coli XL-1 Blue (Stratagene, La Jolla, CA) during an incubation of 4 hrs at 37°C under non-shaking conditions. Next, 8 ml of Super broth (SB: 3% (w/v) peptone-140.2% (w/v) yeast extract-1% (w/v) 3-(N-Morpholino)propanesulfonic acid (MOPS; Sigma; pH 7.0) were added, supplemented with carbobin (20 µg/ml) and tetracycline (10 µg/ml). Serial dilutions (10^-10^-4) were plated to determine the number of phages that were eluted. The infected E.coli cells were pelleted by centrifugation (15 min, 2500 x g), resuspended in 1 ml SB and plated on large (150 mm) plates (100 µg/ml carbobin, 1% (w/v) Glucose) for overnight re-amplification at 37°C. The next day, the cells were scraped off the plate. To resurface the phages, the resuspended cells were diluted to an OD660 of 0.3 in 5 ml SB/10 µg/ml tetracycline/50 µg/ml carbobin, and incubated for 1 hour at 37°C. They were then diluted 1:10 in 10 ml of SB/10 µg/ml tetracycline/50 µg/ml carbobin and 100 µl of helper phage VCS-M13 (10^6 phage forming units/ml) was added directly. The culture was incubated for 1.5 hrs at 37°C (45 min non-shaking, 45 min shaking). The volume was then increased to 40 ml and kanamycin (70 µg/ml) was added. After overnight growth at 30°C the cells were pelleted and the supernatant, containing the phages, was collected. The phages were then precipitated by incubation for 30 minutes on ice with 4% (w/v) PEG 8000 and 3% (w/v) NaCl. The precipitated phages were pelleted (30 min, 14,000 x g, 4°C) and the supernatant discarded. The pellet was resuspended in 1 ml of PBS/1% (w/v) BSA and centrifuged (5 min, 14,000 x g). The supernatant was used for the next round of panning. After four rounds of panning individual colonies were grown and the phages were prepared as described above for screening in GNA-capture ELISA (see below).

**ELISA.** GNA-capture ELISA: Falcon MicroTest III 96-well plates were coated with GNA and blocked as described above. After washing, OG-P1h (1.5), NP-P1h (1:5) or gp120h (1 µg/ml) diluted in 0.4% (w/v) NFDM/0.05% (w/v) gelatine in PBS were added to the plates for 1 hr at RT. Next, the plates were incubated with phage-containing bacterial supernatant or precipitated phages for 1.5 hrs at RT. For competition experiments 100 µg/ml scFv4 was added during phage incubation. Bound phages were detected with HRP-labeled mouse anti-M13 gIg and tetra-methyl-benzidine (TMB) substrate (Merck, Darmstadt, Germany). The color development reaction was stopped by addition of an equal volume of 0.2M H2SO4 and absorbance was measured at 450nm.

Ag-capture ELISA: Falcon MicroTest III 96-well plates were coated with mAb ID-gp120-17 (5 µg/ml) or pAb anti-IgV (5 µg/ml) in PBS and blocked with 4% (w/v) NFDM/0.5% gelatine in PBS. Antigen capturing, phage incubation and detection were performed as described above. Direct Ag coating: Falcon MicroTest III 96-well plates were coated directly with 2 µg/ml of Ag in PBS overnight at RT. Blocking, phage incubation and detection were performed as described above.

**DNA analysis.** Nucleic 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). The primer that was used for sequencing from the 3' end was 5'-TTG GAC TCA TAT GAT GGT GAT GAC CAC TAA AG-3' (5'-ATT AAC CCT CAC TAA AG-3') was used for sequencing from the 5' end of the heavy chain. All primers were made by Gibco-BRL. MacVector software (Genetics computer group, San Diego, CA) was used to compare the heavy chain CDR3 amino-acid sequences of the newly isolated clones with those isolated previously from library M (3, 4, 8, 13, 30). For DNA fingerprinting, the heavy chain (Fd) fragment of the positive clones was amplified using the CG1d (5'-GCA TGT ACT AGT TTT GTC ACA AGA CAC TAA GG-3') and VH135 (5'-AGG TGC AGC TGC TGC GAG CGT G) primer-pair. A third (10 µl) of the amplified DNA was digested with 2 U of Bst NI (New England Biolabs, Beverly, MA) during a 16 hrs incubation according to manufacturer's instructions. The restriction digests were analyzed on 2% agarose/TAE gels.

**Results.**

**Preparation and characterization of panning antigen.** To solubilize membrane associated proteins, the non-ionic detergent n-octyl β-D-glucopyranoside (OG) was used at a concentration of 0.5% (w/v) (17mM). RK13 cells infected with rVV expressing the HIV-1env Proteins were lysed, and the cleared lysate was fractionated on an ACA ultrigel-34 gel-filtration column, equilibrated in 0.05% (w/v) OG (1.7mM). The presence of HIV-1 env in the fractions was determined by means of GNA-capture ELISA. The turbidity (vesicle formation) of the fractions was measured at A280. This revealed two HIV-1 env containing peaks, which were isolated by elution from the column, and thus represent the protein complexes that were excuated from the column, i.e. larger than the cut-off: 750 kD (Fig. 3.1A).

The fractions of the first peak were pooled and referred to as OG-P1h. To determine if the HIV-1 env
was associated with the vesicles, OG-P1IH was analyzed by flow cytometry. The vesicles were stained with AnnexinV (AnxV), indicative of the presence of the phospholipid phosphatidyl serine, and IgG1b12, directed against the CD4 binding domain of HIV-1 Env. Double positive vesicles were observed (Fig. 3.1B). Preincubation with sCD4 selectively inhibited the IgG1b12 signal, indicating Env-specific staining (Fig. 3.1C). Preliminary analysis of OG-P1IH with a confocal microscope revealed a homogeneous population of spherical particles with an average diameter of 0.5 μm (data not shown).

The second peak, falling within the fractionation range of the column and most likely containing the monomeric Env, was also collected and referred to as OG-P2IH. Both OG-P1IH and OG-P2IH were subjected to western blot analysis with anti-HIV-1 Env antiserum from rabbits (Fig. 3.1A insert). The OG-P1IH preparation predominantly contained gp160 with a varying amount of gp120. In Fig. 3.1A (insert) a OG-P1IH is shown that contained a relative large amount of gp120. The OG-P2IH preparations contained both gp160 and gp120.

**Phage selection**

Phage display library M was panned against OG-P1IH in a detergent background of either 0.05% (w/v) OG (gel-filtration column concentration) or 0.5% (w/v) NP-40 (NP-P1IH). NP-40 was used here to disrupt the vesicles. The antigens were presented in GNA-coated ELISA wells. Based on the number of phages eluted from the wells the degree of amplification was determined. After 4 rounds, a 6-fold amplification was observed for the OG-P1IH panning and a 5-fold amplification for the NP-P1IH panning. From the 4th round of panning monoclonal phages were grown from 75 individual colonies from each panning experiment and screened in an ELISA for reactivity with the panning antigens. In the ELISA, GNA alone was used as a negative control (representative ELISA results are shown in Fig. 3.2A). From the OG-P1IH panning, 70 clones (93.3%) were considered positive, from the NP-P1IH panning, 66 clones (88.0%).

**DNA fingerprint- and sequence analysis of positive clones**

The restriction enzyme Bst N1, which frequently cuts in the human γ1 heavy chain, was used to digest the DNA of the amplified heavy-chain from the positive clones (31). Eleven distinct restriction patterns (fingerprints) could be observed (Fig. 3.2B). The clones were grouped according to the resulting DNA fingerprints and the frequency of the clones in the different panning experiments was determined (Fig. 3.2C).

Where possible, at least three clones (preferably originating from different panning strategies) of each fingerprint group were sequenced. The clones were characterized on the basis of their heavy chain complementarity-determining region 3 (HCDR3) and compared with clones previously isolated from library M (Fig. 3.2C). From the OG-P1IH panning, 70 clones (93.3%) were considered positive, from the NP-P1IH panning, 66 clones (88.0%).

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Figure 3.2: Phages were prepared from clones from the fourth round of selection and tested in a GNA-capture ELISA. A) Representative clones from the OG-P1 (left panel) and NP-P1 (right panel) paning are shown. Phage Abs were tested on the panning antigens (solid bars), monomeric gp120 (hatched bars) and GNA alone (open bars). As positive and negative controls, phage b12 (b12) and wild-type (wt) phages respectively were included in each screening. B) DNA was isolated from selected positive clones, the Fd fragment amplified and digested with restriction enzyme BstN1, HaeIII and TaqI and analyzed on a 2% agarose gel. As a marker X174/HaeIII was used (M). C) The clones were grouped according to their DNA fingerprints and the frequencies in both pannings were determined. Representative positive clones, derived from each fingerprint group, were sequenced. The amino-acid sequence of the heavy chain complementarity-determining region 3 (CDR3) and the flanking framework regions (FR3 and FR4) were deduced. Dashes indicate sequence identity to first sequence in group. Sequence numbering is according to Kabat. Underlined clones were selected as prototypic clones for further characterization.

<table>
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<th>Frequency</th>
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<tr>
<td></td>
<td>OG</td>
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</tr>
<tr>
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M8B (275; 2.6%) (4). The NP-P1mt also selected for MOG2-like and MOG3-like clones at frequencies of 14/75 (18.7%) and 4/75 (5.3%), respectively. Furthermore, this panning selected for three other clones with novel HCDR3 sequences, termed MNP1 through MNP3. MNP1 and MNP3 were present as single clones (1.3%), whereas MNP2 was present at a frequency of 7/75 (9.3%). None of the panning strategies selected for clones related to the relatively strongly neutralizing antibody b12 (9), although this clone was able to bind to the panning antigen (see Fig. 3.1C and 3.2A).
Table 3.1: Summary of ELISA reactivity of isolated clones

<table>
<thead>
<tr>
<th>Capt</th>
<th>Ag</th>
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<td></td>
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<td>MNP</td>
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<tr>
<td>ID-17</td>
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<td>C1-inh</td>
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Capt= capturing agent, Ag= antigen, wt=wild type phage
++=strong reactivity, +=weak reactivity, -=no reactivity

Specificity of newly isolated Fab clones

To further characterize the specificity of the newly isolated clones, a prototypic clone from each group was selected and phages were grown. Precipitated phages were tested in a number of Ag-capture-ELISAs (Table 3.1). To exclude reactivity to host-cell derived antigens HeLa cells, instead of RK13 cells, were used to prepare OG-P1\textsubscript{IW}. All clones remained reactive with HeLa cell derived OG-P1\textsubscript{1H}. Upon NP-40 treatment the reactivity of all clones increased, except for the MOG1 clone, which lost its reactivity.

To determine HIV-1 Env specificity, mAb ID-gp120-17, specific for a conformational epitope in the C1-domain of gp120, was used as capturing agent. This revealed that two new clones (MOG2 and MOG3) derived from the OG-P1\textsubscript{1H} panning and two new clones (MNP2 and MNP3) derived from the NP-P1\textsubscript{1H} panning, were HIV-1 specific. Preliminary epitope mapping studies (data not shown) indicate that none of the newly isolated HIV-1 specific clones were directed against the CD4 binding domain. To verify that the clones were directed against conserved regions on HIV-1 gp120, the clones were tested for reactivity with gp120\textsubscript{LAv} or gp120\textsubscript{UV} (Table 3.1). None of the novel clones bound to gp120\textsubscript{LAv} nor gp120\textsubscript{UV}, whereas the b12 and s8 clones did.

To determine the specificity for vaccinia derived proteins, precipitated phages were tested in an ELISA for reactivity with NP-P1\textsubscript{1H} captured by a Vaccinia specific rabbit pAb. The highly dominant MOG1 clone, derived from the OG-P1\textsubscript{1H} panning, reacted with Vaccinia proteins, as did the MNP6 clone derived from the NP-P1\textsubscript{1H} panning.

In total 11 clones (14.7%) from the OG-P1\textsubscript{1H} panning were HIV-1 specific, whereas 59 clones (78.6%) were specific for vaccinia. For the NP-P1\textsubscript{1H} panning 65 clones (86.7%) were HIV-1 specific, whereas 1 clone (1.3%) was specific for vaccinia (Fig. 3.3).

Discussion

Traditional selection strategies for the isolation specific Abs often depend on the availability of purified antigens. Some classes of proteins however, like integral membrane proteins, are not easy to purify and thus require intricate selection strategies. Here we describe an elegant approach for the generation of panning antigens suitable for the selection of phage libraries against membrane proteins. In this study the envelope glycoprotein complex (Env) of HIV-1 was used as a model for a type-1 integral membrane protein. Env was expressed on membranes by infecting cells with recombinant vaccinia virus encoding for gp160.

The first step in our approach was the solubilization of the membrane protein of interest from the native membrane. The mild non-ionic detergent OG has been successfully used for this purpose (38). OG differs from most other non-ionic detergents in that it has a relative high critical micellar concentration (cmc), which makes removal of detergent easier. The concentration of OG would ideally be such that membrane-associated proteins are solubilized including phospholipids, but non-covalent protein interactions (e.g. gp120-gp41) are maintained. In this respect Helenius et al. (23) reported the preservation of the non-covalent interaction between the Semliki Forest Virus (SFV) Env protein and capsid protein in 30 mM OG (0.68%). Additionally, Heinz et al. (21) reported that almost half of the phospholipid content of the tick-borne Encephalitis (TBE) virus was still associated with the TBE Env after treatment with 1% OG (34 mM). Furthermore, Patenostre et al. (38) reported the minimal concentration of OG (14 mM; 0.41%) needed to solubilize all viral Env proteins from VSV virosomes. The
The MOG2 and MOG3 clones seem to be directed to agreement with current views (17, 47, 52) or the other membrane context (oligomeric forms), which is in whereas NP-P1IHI selected for 20/65 (30.8%). This There were, however, some qualitative differences. OG-soluble (NP-P1IHI) Ags selected for HIV-1 specific Fabs. (Table 3.1) as a consequence of NP-40 treatment Abs associated proteins. The increase in binding of the phage Abs indicates that not all Env is incorporated in the right orientation. For example, for HIV-1 Env expression it was reported that the majority of Env was retained in the ER and subsequently degraded (6, 11, 50). In vivo, unprocessed gp160 or monomeric gp120, are thought to play a role as decay antigens, exposing non-relevant epitopes (35). Similarly the intracellular contaminants in the panning Ag would also serve as decay for the selection procedure and should thus be avoided. To get rid of these contaminants the OG-solubilized proteins were fractionated by gel-filtration. Simultaneously, the concentration of OG was reduced to 0.05% OG (column concentration), which is below the critical micellar concentration (cmc) of OG. As a result the lipids still associated with the solubilized integral membrane proteins re-associate to form high molecular weight vesicles along the column, trapping the membrane-associated proteins, and separating them from the soluble, non-membrane associated proteins. The increase in binding of the phage Abs (Table 3.1) as a consequence of NP-40 treatment indicates that not all Env is incorporated in the right orientation.

Both membrane-associated (OG-P1IH) and soluble (NP-P1IH) Ags selected for HIV-1 specific Fabs. There were, however, some qualitative differences. OG-P1IH selected for 1/11 (9.1%) CD4bd specific clone, whereas NP-P1IH selected for 20/65 (30.8%). This indicates that the CD4bd is either less exposed in the membrane context (oligomeric forms), which is in agreement with current views (17, 47, 52) or the other epitopes are immuno-dominant in the membrane context. The MOG2 and MOG3 clones seem to be directed to epitopes that are well exposed on both types of antigen. In the OG-P1IH panning, we found a considerable enrichment for a vaccinia specific Fab (MOG1). This is probably a reflection of the high prevalence of vaccinia-derived proteins on the vesicles and the immune-state of the donor. Conversely, the NP-40 treated panning Ag only selected for a single vaccinia-specific clone (MNP1). The fact that NP-P1IH did not select for MOG1 combined with the observation that MOG1 binding to P1IH in the GNA-capture ELISA was abolished after NP-40 treatment, suggests that the MOG1 Ag does not bind to GNA directly, but is captured via its association with the vesicles. This suggests that with our strategy capturing of an Ag in trans, e.g. mAbs to other integral membrane proteins in the preparation, is also possible. This would be preferable if no Ag-specific capturing agents are available or if the membrane context is important for antigenicity. Studies that have investigated the parameters that affect selection procedures suggest that the density of the Ag can greatly influence the success of such procedures (28, 33). Experimental expression systems like the one we used, are often chosen for their high expression levels and thus seem better candidates for our type of selection. However, with fine-tuning of our strategy it could be possible to select for naturally expressed cell-surface proteins.

HIV-1 Env-specific Fab fragments have been isolated from phage antibody fragment libraries using purified Env subunits (3, 4, 8, 13). However, the neutralizing ability of the Env-specific Fabs was shown to correlate with the affinity to the mature oligomeric configuration, rather than affinity to functionally irrelevant forms of Env, i.e. monomeric or unprocessed Env (17, 37, 40, 43). Concentrated virions expressing the mature trimeric configuration enriched for the relatively strong neutralizing b12 clone (36). Apparently, the successful rescue of recombinant human Fab fragments with HIV-1 neutralizing activity from a phage display library largely depends on the presentation of envelope proteins in a physiological relevant configuration. As OG-P1IH failed to select for the relatively strong neutralizing b12 clone, present in the library, it suggests that OG-P1IH does not represent the mature oligomeric Env. We think this is probably due to the predominance of gp160 (uncleaved Env) present in OG-P1IH, which is likely the result of saturation of the cellular furin-like proteases responsible for Env cleavage due to the high levels of protein expression. Over-expressing furin for the generation of OG-P1IH should prove more successful (5). Another explanation for the inability of OG-P1IH to select for b12 is that its epitope is not exposed on HIV-1 Env. This, however, is contradicted by the facts that phage b12 could bind to OG/NP-P1IH in GNA-capture ELISA, that IgG1b12 could bind to P1IH in flow cytometry and that the HIV-1CMI-172.B-ai isolate, from which HIV-1 Env was cloned, could be neutralized by IgG1b12 (IC50 = 6.25 µg/ml, unpublished data).

Another observation is that previously unknown clones could still be isolated from a relatively well-characterized library. The reason for isolating new clones, could simply be a technical one. The solid state
amplification used in this study, as compared to liquid state amplification (8), is more favorable for clones with a growth disadvantage. Alternatively a screening procedure based on phages may detect additional positive clones because of avidity instead of affinity. Finally the origin of the Env used is this panning, a prototypic RS primary isolate, could have influenced the selected repertoire as well.

In conclusion, we describe a simple approach for the generation of panning antigens based on the solubilization and reconstitution of integral membrane proteins. With this method we could select known and novel HIV-1 Env-specific Abs from a well-characterized phage-display library made from an HIV-1 seropositive donor. We suggest this approach may be applicable for the selection of Abs against membrane protein.

Acknowledgement
We thank Martin de Boer for assistance in DNA sequencing. We acknowledge Dr. Peter Hordijk for confocal microscopic analysis. We are grateful to Dr. Paul Parren for critically reading the manuscript.

References


