Neutralizing antibodies to the HIV-1 envelope glycoproteins

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Publication date
2004

Chapter 3

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

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J. Immunol. Methods (261): 37-48' 2002

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_{IHI} 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-$P1_{IHI}$, 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-$P1_{IHI}$ vesicles disrupted by Nonidet P-40 (NP-$P1_{IHI}$). Both membrane-associated and soluble Ags selected for vaccinia-specific clones (OG-$P1_{IHI}$: 59/75 and NP-$P1_{IHI}$: 1/75), and HIV-1-specifc clones (OG-$P1_{IHI}$: 11/75 and NP-$P1_{IHI}$: 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 these methods provides a major challenge, in particular when the target Ag is expressed at a low level.

The human immunodeficiency virus type 1 (HIV-1) envelope protein complex (Env) is synthesized as a precursor glycoprotein gp160, which oligomerizes in the ER (14, 29). In a Golgi or post-Golgi compartment, gp160 is cleaved by a cellular furin-type protease into the gp120 and gp41 subunits, which stay non-covalently associated (2, 19, 41). The resulting mature functional Env, i.e. $(gp120\text{-}gp41)_3$, is then expressed on the surface of virions and infected cells. As intact virions are difficult to work with (36) and expression of functional Env by infected cells only occurs at low levels, we applied a novel panning strategy to select phage-expressed antibodies against Env.

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 antisera against gp160/gp120 of HIV-1_{W61D}, produced in CHO cells (CLB-W61D, produced in our laboratory), mouse ID-120-17 monoclonal (m)Ab, directed to a conformational epitope in the C1 domain of HIV-1 gp120 (unpublished data), horseradish peroxidase (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-anti rabbit IgG (Pierce, Rockford, IL). Biotinylation of IgG1b12 was done by incubation with Sulfosuccinimidyl-6-(biotinamido) Hexanoate (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 $gp120_{MN}$ and $gp120_{IIIB}$, (contributed by Immunodiagnostics, Woburn, MA), soluble (s)CD4 (contributed by Dr. R. Sweet). C_1 inhibitor was obtained from the CLB (Amsterdam, The Netherlands).

Viruses and Cell lines. RK13 and HeLa cells 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 μg/ml) at 37°C in 5% CO_2. A recombinant vaccinia virus (rVV) expressing the HIV-1_{IHI} *env* gene (rVV_{IHI}), 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 cm^2 flasks, were infected with 5 M.O.I. rVV_{IHI}. 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% (w/v) n-octyl β-D-glucopyranoside (OG; Sigma,

St. Louis, MO)/150 mM NaCl/200 μM PMSF/80 μM Pepstatin in PBS (lysis buffer). After removal of cell-debris (10 min, 2050 x g, 4°C), the lysate was applied on an ACA ultrogel-34 gel-filtration column (Biosepra, Cergy-Saint-Christophe, France). The column was equilibrated in lysis buffer except for an OG concentration of 0.05% (w/v). Fractions were collected and assessed for Env content by means of GNA-capture ELISA (see below). The presence of vesicles was measured by light scattering (turbidity) A800nm. Two Env containing peaks could be observed (Fig.1A). The fractions comprising each peak were pooled and referred to as, OG-$P1_{IHI}$ and OG-$P2_{IHI}$.

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 (Amersham 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 $CaCl_2$/2mM $MgCl_2$/0.1% (w/v) BSA/0.02% (w/v) NaN_3 at pH 7.2 (FACS buffer) and incubated with 1 μg/ml of biotinylated mAb IgG1b12 for 30 min at 37°C. For sCD4 inhibition the vesicles were first pretreated for 15 minutes with 50 or 5 μg/ml of sCD4 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 (AnxV-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 FACSCalibur flow cytometer with CellQuest software (Becton Dickinson). A total of 30,000 events were measured per sample.

Panning of the antibody phage-display library. A Fab display library, constructed on the surface of 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) not-fat dry milk (NFDM; Campina, Eindhoven, The Netherlands) and 0.5% (w/v) gelatine (Merck, Darmstad, Germany) for 1 hr at RT and washed with PBS. OG-$P1_{IHI}$ 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-$P1_{IHI}$) and incubated for 1 hr at 37°C. Per 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 45 minutes 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 carbenicillin (20 μg/ml) and tetracycline (10 μg/ml). Serial dilutions (10^{-3},10^{-4} and 10^{-5}) were plated to determine the number of phages that were eluted. The infected *E.coli* cells were pelleted by centrifugation (10 min, 2500 x g), resuspended in 1 ml SB and plated on four large (150 mm^2) plates (100 μg/ml carbenicillin, 1% (w/v) Glucose) for overnight re-amplification at 30°C. The next day, the cells were scraped off the plate. To rescue the phages, the resuspended cells were diluted to an OD_{600} of 0.3 in 5 ml SB/10 μg/ml tetracycline/50 μg/ml carbenicillin, 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 carbenicillin and 100 μl of helper phage VCS-M13 (10^{12} plaque forming units) were added. 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 phages were prepared as discussed 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-$P1_{IHI}$ (1:5), NP-$P1_{IHI}$ (1:5) or $gp120_{MN}$ (1 μg/ml) diluted in 0.4% (w/v) NFDM/0.05% (w/v) gelatin 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 sCD4 was added during phage incubation. Bound phages were detected with HRP-labeled mouse anti-M13 IgG and tetra-methyl-benzidine (TMB) substrate (Merck, Darmstadt, Germany). The color development reaction was stopped by addition of an equal volume of 0.2M H_2SO_4 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-VV (5 μg/ml) in PBS and blocked with 4% (w/v) NFDM/ 0.5% gelatin 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 SeqGz (5'-GAA GTA GTC CTT GAC CAG-3'), which hybridizes to the constant domains of the γ1 heavy chain. Primer T3 (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, 36). 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 TTT GG-3') and VH135 (5'-AGG TGC AGC TGC TCG AGT CTG 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-1_{IHI} Env proteins were lysed, and the cleared lysate was fractionated on an ACA ultrogel-34 gel-filtration column, equilibrated in 0.05% (w/v) OG (1.7mM). The presence of HIV-1_{IHI} Env in the fractions was determined by means of GNA-capture ELISA. The turbidity (vesicle formation) of the fractions was measured at A_{800}. This revealed two HIV-1_{IHI} Env containing peaks, the first of which co-eluted with the turbid fractions and the void volume of the column, and thus represent the protein complexes that were excluded 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-$P1_{IHI}$. To determine if the HIV-1_{IHI} Env

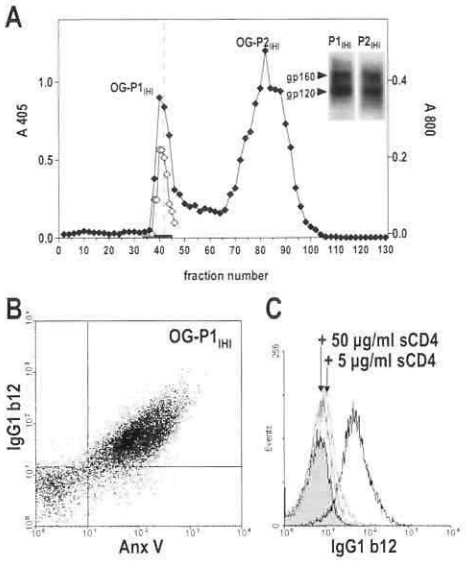

Figure 3.1: Preparation of vesicles expressing the Env complex of HIV-1_{IHI}. HeLa/RK13 cells infected with rVV_{IHI} were lysed in 0.5% OG (w/v). After removal of cell-debris the lysates were applied to an ACA ultrogel-34 gel-filtration column equilibrated in 0.05% (w/v) OG and (500 µl) fractions were collected. **A**) Env content in the fractions was assessed by means of GNA-capture ELISA with rabbit antiserum CLB-W61D (-♦-). Turbidity (A_{800}; -◊-) was measured to detect vesicles. Fractions 38-44 (indicated by the solid bar) and fraction 72-92 (indicated by the open bar) were pooled and referred to as OG-$P1_{IHI}$ and OG-$P2_{IHI}$ respectively. Both OG-$P1_{IHI}$ and OG-$P2_{IHI}$ were subjected to western blot analysis and visualized with rabbit anti-Env antibodies (insert). **B**) OG-$P1_{IHI}$ was stained with biotinylated IgG1b12 followed by Streptavidin-PE versus AnnexinV-FITC (AnxV) and measured by flow cytometry. **C**) AnxV positive vesicles were gated and the effect of preincubation with sCD4 (50 or 5 µg/ml; grey lines) on IgG1b12 binding (black line) was investigated (Streptavidin-PE alone; solid histogram).

was associated with the vesicles, OG-$P1_{IHI}$ 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_{IHI} 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-$P1_{IHI}$ 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-$P2_{IHI}$. Both OG-$P1_{IHI}$ and OG-$P2_{IHI}$ were subjected to western blot analysis with anti-HIV-1 Env antiserum from rabbits (**Fig. 3.1A insert**). The OG-$P1_{IHI}$ preparation predominantly contained gp160 with a varying amount of gp120. In **Fig. 3.1A** (insert) a OG-$P1_{IHI}$ is shown that contained a relative large amount of gp120. The OG-$P2_{IHI}$ preparations contained both gp160 and gp120.

Phage selection

Phage display library M was panned against OG-$P1_{IHI}$ in a detergent background of either 0.05% (w/v) OG (gel-filtration column concentration) or 0.5% (w/v) NP-40 (NP-$P1_{IHI}$). 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-$P1_{IHI}$ panning and a 5-fold amplification for the NP-$P1_{IHI}$ 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-$P1_{IHI}$ panning, 70 clones (93.3%) were considered positive, from the NP-$P1_{IHI}$ 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**) (3, 4, 8, 13, 36).

The panning experiment using OG-$P1_{IHI}$ selected two clones that were related to the previously isolated clones, b8, a gp120 CD4-binding domain (CD4bd) specific Fab (3), and T3, a gp41 cluster II specific Fab (4). Additionally three clones with novel HCDR3 sequences, termed MOG1 through MOG3, were selected. The OG-$P1_{IHI}$ mainly selected for MOG1-like clones, as these were present at relative high frequencies of 59/75 (78.7 %). MOG2- and MOG3-like clones were present at frequencies of 6/75 (8.0 %) and 3/75 (4.0 %), respectively.

The NP-$P1_{IHI}$ also selected for clones related to the b8 and T3 clones, but at higher frequencies, 16/75 (21.3%) and 17/75 (22.7%) respectively, compared to OG-$P1_{IHI}$. Additionally, previously identified gp120 CD4bd specific clones b5 (3/75; 4.0%) and s8 (1/75; 1.3%) (3) were also selected, as was gp41 cluster I specific clone

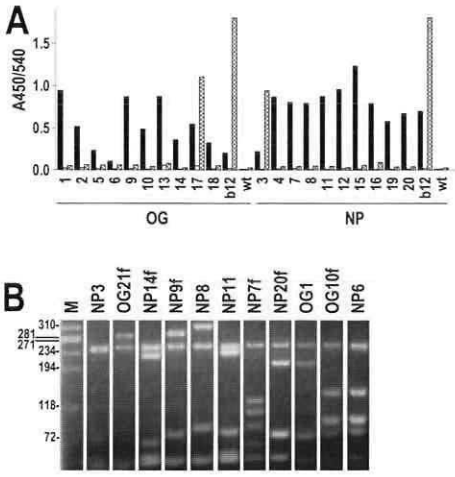

Figure 3.2: Phages were prepared from clones from the forth round of selection and tested in a GNA-capture ELISA. **A**) Representative clones from the OG-P1$_{IHI}$ panning (OG; left panel) and NP-P1$_{IHI}$ panning (NP; right panel) are shown. Phage Abs were tested on the panning antigens (solid bars), monomeric gp120$_{MN}$ (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 *Bst* NI, *Hae* III and *Taq* I and analyzed on a 2% agarose gel. As a marker φX174/*Hae* III 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.

C

Name	Clone	Frequency		Heavy-Chain		
		OG	NP	FR3	CDR3	FR4
				9	10	
				01234	567890abcdefghijklmnop12	3456
b5	NP3f	-	3	YYCAT	KYPRYFDMMAGVRNHFYMDV	WGQG
	NP20f			-----	----------	----
	NP30f			-----	----------	----
b8	OG17	1	16	YYCAR	DIGLKAEHYDILTAYGPDY	WGQG
	NP3			-----	-----G----------	----
	NP35f			-----	----------	----
s8	NP9f	-	1	YYCAR	GRRLVTFGGVVAGGNI	WGQG
M8B	NP14f	-	2	YYCTG	VVQAVQLSDGRNWLDP	WGQG
	NP28f			-----	----------	----
T3	OG35f	1	17	YYCAR	GPNFYSGYGRRHHFSALDV	WGKG
	NP6			-----	----------	----
	NP18f			-----	----------	----
MOG1	OG1	59	-	YYCAR	AVRGLGAFDV	WGQG
	OG2			-----	----------	----
	OG18			-----	----------	----
	OG1f			-----	----------	----
MOG2	OG9	6	14	YYCAL	RIKYSPGHVGNFDY	WGEG
	NP4			-----	----------	----
	NP8f			-----	----------	----
	OG21f			-----	----------	----
MOG3	OG5	3	4	YYCAR	DEGPQWDSGEYDVGSYFDY	WGQG
	OG14			-----	----------	----
	OG10f			-----	------EA----------	----
	NP22f			-----	----------H--S	----
MNP1	NP8	-	1	YYCAA	RSEPVWADYGDYPGETRDFNYMDV	WGKG
MNP2	NP11	-	7	YYCAR	GDYDFWSGEAGPDINWFDP	WGQG
	NP14			-----	----------	----
	NP26f			-----	----------	----
	NP47f			-----	----------	----
MNP3	NP7f	-	1	YFCVV	RLKYLPGQTSFFDF	WGQG
		70	66			

M8B (2/75; 2.6%) (4). The NP-P1$_{IHI}$ 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

Capt	Ag	Clones								
		MOG			MNP			controls		
		1	2	3	1	2	3	s8	b12	wt
ID-17	NP-P1$_{IHI}$	-	++	++	-	+	+	++	++	-
a-VV	NP-P1$_{IHI}$	++	-	-	+	-	-	-	-	-
	NP-P1$_{IHI}$	-	++	++	++	++	++	++	++	-
	OG-P1$_{IHI}$	++	+	+	+	+	+	+	+	-
GNA	gp120$_{MN}$	-	-	-	-	-	-	++	++	-
	gp120$_{LAV}$	-	-	-	-	-	-	++	++	-
	C1-inh	-	-	-	-	-	-	-	-	-

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$_{IHI}$. All clones remained reactive with HeLa cell derived OG-P1$_{IHI}$. 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$_{IHI}$ panning and two new clones (MNP2 and MNP3) derived from the NP-P1$_{IHI}$ 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$_{LAV}$ or gp120$_{MN}$ (**Table 3.1**). None of the novel clones bound to gp120$_{LAV}$ nor gp120$_{MN}$, 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$_{IHI}$ captured by a Vaccinia specific rabbit pAb. The highly dominant MOG1 clone, derived from the OG-P1$_{IHI}$ panning, reacted with Vaccinia proteins, as did the MNP6 clone derived from the NP-P1$_{IHI}$ panning.

In total 11 clones (14.7%) from the OG-P1$_{IHI}$ panning were HIV-1-specific, whereas 59 clones (78.6%) were specific for vaccinia. For the NP-P1$_{IHI}$ 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 (0.88%). 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, Paternostre et al. (38) reported the minimal concentration of OG (14 mM; 0.41%) needed to solubilize all viral Env proteins from VSV virosomes. The

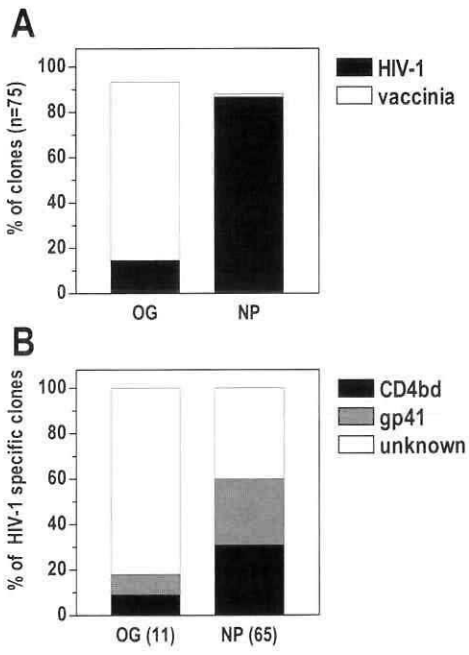

Figure 3.3: The frequency of clones, indicated by the specificity, in **A**) the total phage population and **B**) the HIV-1 specific population, after selecting the library for four rounds of panning against the antigen indicated (OG = OG-P1$_{IHI}$, NP = NP-P1$_{IHI}$). The number of clones used for the data represented in each bar is given in parentheses.

authors argued that minimizing the detergent concentration was a critical step in the successful functional reconstitution of the solubilized Env. Taking all these factors into consideration we choose an OG concentration of 0.5% (17mM). Indeed the Env, derived from a 0.5% OG lysate, could re-associate into phospholipid containing vesicles (**Fig. 3.1B**), indicating that phospholipids were still associated with the proteins. As the presence of gp120 in this membrane associated Env could be demonstrated (**Fig. 3.1A insert**) the non-covalent interactions were at least partly preserved.

Membrane proteins normally represent a very small portion of the total cellular protein content. Contamination by other cellular proteins liberated by the detergent could therefor represent targets for non-relevant phages and should thus be avoided. Capturing the antigen of interest from a cell-lysate by Ag-specific mAbs, has been successful (42, 44). In our study we used the lectin, *Galanthus nivalis* agglutinin (GNA), as a capturing agent. Although lectins are generally less specific than mAbs, GNA does show considerable specificity for HIV-1 Env (18, 24, 30). In general, lectins make attractive Ag-capturing agents for panning experiments, as the carbohydrate structures, to which they bind, are typically considered immuno-silent. Lectins therefor are not likely to mask relevant epitopes and consequently biasing the selected repertoire.

High expression levels introduce other contaminants due to saturation of the cellular processing machinery. 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 decoy antigens, exposing non-relevant epitopes (35). Similarly the intracellular contaminants in the panning Ag would also serve as decoy for the selection procedure and should thus be removed. 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-$P1_{IHI}$) and soluble (NP-$P1_{IHI}$) Ags selected for HIV-1 specific Fabs. There were, however, some qualitative differences. OG-$P1_{IHI}$ selected for 1/11 (9.1%) CD4bd specific clone, whereas NP-$P1_{IHI}$ 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-$P1_{IHI}$ 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-$P1_{IHI}$ did not select for MOG1 combined with the observation that MOG1 binding to $P1_{IHI}$ 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-$P1_{IHI}$ failed to select for the relatively strong neutralizing b12 clone, present in the library, it suggests that OG-$P1_{IHI}$ does not represent the mature oligomeric Env. We think this is probably due to the predominance of gp160 (uncleaved Env) present in OG-$P1_{IHI}$, 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-$P1_{IHI}$ should prove more successful (5). Another explanation for the inability of OG-$P1_{IHI}$ to select for b12 is that its epitope is not exposed on HIV-1_{IHI} Env. This, however, is contradicted by the facts that phage b12 could bind to OG-/NP-$P1_{IHI}$ in GNA-capture ELISA, that IgG1b12 could bind to $P1_{IHI}$ in flow cytometry and that the HIV-$1_{ACH-172-B-al}$ isolate, from which HIV-1_{IHI} Env was cloned, could be neutralized by IgG1b12 (IC_{90} = 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 R5 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.

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