

## Short Communication

# Array assessment of phage-displayed peptide mimics of Human Immunodeficiency Virus type 1 gp41 immunodominant epitope: Binding to antibodies of infected individuals

Marie-Claire Arnaud<sup>1</sup>, Tatyana Gazarian<sup>2</sup>, Yadira Palacios Rodriguez<sup>3</sup>, Karlen Gazarian<sup>3</sup> and Vehary Sakanyan<sup>1,4</sup>

<sup>1</sup>Biotechnologie, Biocatalyse, Biorégulation, CNRS UMR 6204, Université de Nantes, Nantes, France

<sup>2</sup>Departamento de Salud Pública, Facultad de Medicina, Universidad Nacional, Autónoma de México, México, México

<sup>3</sup>Departamento de Biología Molecular y Biotecnología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, México, México

<sup>4</sup>ProtNeteomix, Université de Nantes, Nantes, France

An array of phage-displayed mimetic peptides representing 18 sequences from the HIV-1 gp41 immunodominant epitope was fabricated on a nitrocellulose membrane. It used to compare the antibody-binding affinity of the peptide and to monitor the immune response of four patients prior to and after the initiation of Highly Active Anti-Retroviral Therapy in parallel assays with several probes using near-infrared fluorescence detection. The proposed multiplexed approach is highly sensitive, consumes less sample and can be used to analyze the immune repertoires of virus-infected individuals.

**Keywords:** Gp41 epitope mimotopes / HIV-1 / Immune response / Phage display / Protein array

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Miniaturized protein array technology provides many binding reactions in a single assay and opens up new perspectives in the development of antigen/antibody-based diagnostic tools. Indeed, arrays prepared by immobilization in the solid phase, of purified antigens as capture molecules have been successfully used to detect specific antibodies by means of chemiluminescence or visible fluorescence detection [1–5]. Recently, it has been shown that the sensitivity of the detection of interacting partners with proteins arrayed on a NC membrane can be significantly improved by using probes labeled with near-infrared fluorescent dyes [6, 7]. This is an important step towards the identification of low abundance and low affinity antigens in protein complexes or in cell extracts. In view of the development of new applications of array technol-

ogy for biomedical research, there is significant interest in assessing arrays of epitope-mimicking peptides displayed in large phage particles, such as for the analysis of human immune repertoires in virus-infected patients. The transmembrane gp41 protein of the Human Immunodeficiency Virus type 1 (HIV-1) harbors a major immunodominant epitope, CSGKLIIC. This epitope forms a disulfide-bonded loop recognized by the sera of almost all HIV-1 of infected individuals [8–11]. Therefore, the epitope has been used for the immunological diagnosis of HIV-1 [8]. More recently, mimetic peptides of the epitope selected from combinatorial phage-display libraries have been used for studying HIV-1-specific humoral immunity in patients by ELISA using microplate and dot-blot formats [12, 13]. In this study, we have taken advantage of protein array technology to assess the HIV-1-specific immune response to the gp41 major immunodominant epitope in patients, using its peptide mimics selected from a phage display library as antigen. A list of the 18 sequence variations of the major immunodominant epitope of gp41 used is shown in Table 1 (other mimotopes of HIV-1 epitopes selected from the library were not studied here; Gazarian *et al.*, unpublished data).

**Correspondence:** Dr. Vehary Sakanyan, Biotechnologie, Biocatalyse, Biorégulation, CNRS UMR 6204, FRE-CNRS 2230, Université de Nantes, 2 rue de la Houssinière, 44322 Nantes, France  
**E-mail:** vehary.sakanyan@chimbio.univ-nantes.fr  
**Fax:** +33-251125637

**Abbreviations:** HAART, highly active anti-retroviral therapy; HIV-1, human immunodeficiency virus type 1

**Table 1.** Antibody-binding reactivity of phage-displayed mimetic peptides monitored by antigen array and microplate ELISA methods

Phage clone	Amino acid sequence in selected peptides <sup>a)</sup>	Fluorescence intensity at 800 nm, antigen array <sup>b)</sup>	OD <sub>405</sub> nm, ELISA <sup>b), c)</sup>
Selected peptides prior to HAART of patient 1			
1.1.5	LPQQA <b>CLGKLLC</b>	5248/309 (17)	1.49/0.21 (7)
1.1.4	FG <b>CLGKLVCDPY</b>	3740/218 (17)	1.75/0.25 (7)
1.1.1	TP <b>CTYKMTCTTK</b>	2418/144 (17)	1.66/0.24 (7)
1.1.6	LL <b>ACTM<del>K</del>LPCSY</b>	2157/197 (11)	1.43/0.24 (6)
1.1.16	Y <b>QCERKAPCSTY</b>	1874/188 (10)	1.13/0.19 (6)
1.1.8	IG <b>MCKMKAPCAT</b>	1891/145 (13)	0.98/0.20 (5)
1.1.20	LP <b>CHSKYPCIAS</b>	1637/177 (9)	0.58/0.12 (5)
1.1.10	ISM <b>CKEK<del>E</del>VCQT</b>	1835/274 (7)	0.80/0.21 (4)
1.1.26	AL <b>QCQYKVPCLV</b>	577/192 (3)	0.67/0.17 (4)
Selected peptides after 4-week HAART of patient 1			
1.2.18	<b>ACTFKNVCTTKP</b>	2579/188 (14)	1.40/0.36 (4)
1.2.1	H <b>ACTGKLRCTTT</b>	2472/211 (12)	nd <sup>c)</sup>
1.2.2	HLS <b>ICDSKLI<del>C</del>H</b>	1518/138 (11)	nd <sup>c)</sup>
1.2.16	SM <b>CSLKTACTTA</b>	612/77 (8)	nd <sup>c)</sup>
1.2.3	TH <b>QCLGKLVCGV</b>	381/240 (< 2)	nd <sup>c)</sup>
Selected peptides prior to HAART of patient 3			
3.1.23	D <b>VMGKLVCTML</b>	2113/172 (12)	1.36/0.19 (7)
3.1.11	T <b>ICSMKSACTTW</b>	1204/107 (11)	0.76/0.15 (5)
3.1.24	SP <b>NCEGKII<del>C</del>GS</b>	1514/175 (9)	0.77/0.19 (4)
3.1.22	Y <b>GCHSKITCTTF</b>	735/287 (3)	nd <sup>c)</sup>

a) The conserved amino acid residues in selected peptides are shown in bold

b) The antibody-binding prevalence (in parentheses) shows the ratio of the average reactivity data monitored by the two methods using IgG purified from infected patients and noninfected (control) individual as described in the text. The sera of patients 1 and 3 prior to HAART were used for clones 1.1.x and 3.1.x, respectively and the serum of patient 1 after a 12-week HAART was used for clone 1.2.x.

c) nd, not determined.

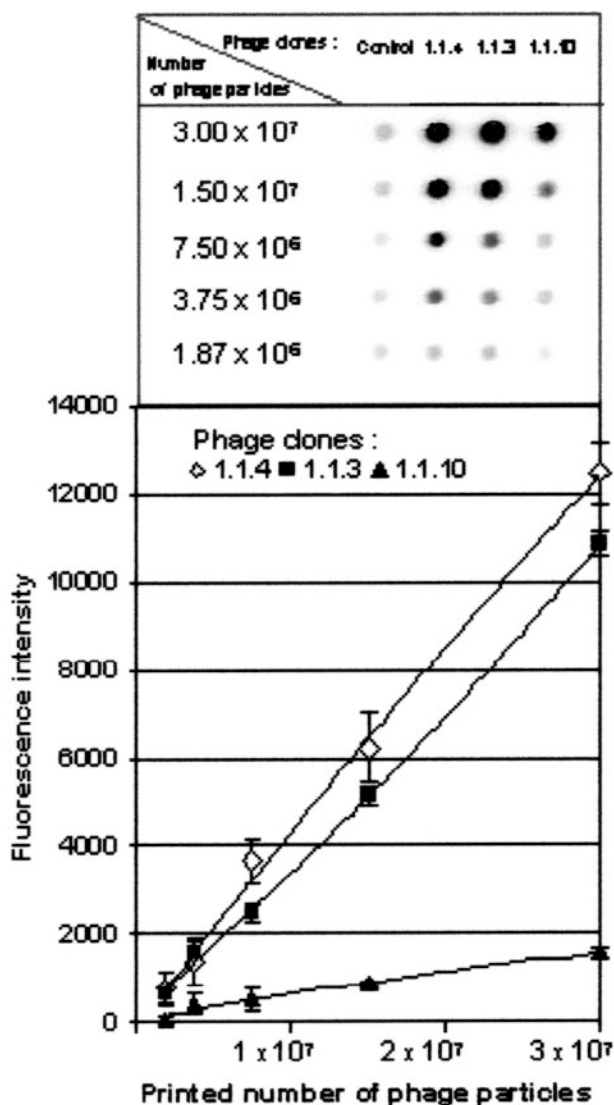
Blood specimens were taken from four infected patients before and after initiation of antiviral therapy in the Gabriel Mancera hospital (MSS, Mexico City, Mexico). The IgG fraction was purified from the sera by affinity chromatography on protein G-agarose (Life Technologies, Gaithersburg, MA, USA). Its purity and concentration were determined with a 2100 bioanalyzer (Agilent Technologies, Massy, France) using a BSA calibration curve. A 12-mer linear M13 phage display library (New England BioLabs, Beverly, MA, USA) was used for the selection of peptide mimics of HIV-1 virus with the sera of infected patients for biopanning (Gazarian *et al.*, unpublished data). In this study, phage clones selected with the serum of patient 1 prior to Highly Active Anti-Retroviral therapy (HAART) are marked as clones 1.1.x. After 4-weeks of HAART they are marked as clones 1.2.x. Clones selected with the serum of patient 3 prior to HAART are marked as clones 3.1.x (see Table 1). The titer of phage particles was carefully ad-

justed to  $1 \times 10^{13}$  PFU/mL in PBS solution in a 96-well microtiter plate and 0.5–1.0 nL of each phage suspension was printed (125  $\mu$ m spot diameter and 500  $\mu$ m space between spots) using a GMS 417 arrayer (MWG Biotech France, Courtaboeuf, France) equipped with 4 steel pins onto a Protran BA83 NC membrane (Schleicher & Schuell Bioscience, Dassel, Germany). If necessary, phage clones were serially diluted 2-fold. Phage particles ( $3.0 \times 10^7$  to  $1.0 \times 10^6$  per spot) were printed on membranes at 18°C and 60% humidity. Membranes with ordered spots of phage clones were used immediately or sealed in a slide box and stored at 4°C until use. Membranes were rinsed in PBS/0.1% Tween-20 for 3 min and incubated in a 5% BSA/PBS blocking solution with gentle shaking for 1 h at 18°C. The blocking solution was removed and membranes were incubated in PBS solution containing a purified IgG fraction (10  $\mu$ g/mL) at 37°C for 1 h (typically binding was performed in 300  $\mu$ L with dif-

ferent IgG probes in parallel experiments). Excess non-bound IgG was removed by 3-fold washing with PBS/0.1% Tween-20 at 18°C and then the membranes were incubated with IRDye 800-labeled anti-human IgG (LI-COR, Courtaboeuf, France) at a final concentration of 0.5 µg/mL at 18°C for 1 h. After three intensive washes in PBS/0.1% Tween-20 at 18°C, membranes were scanned at 800 nm with an Odyssey Imager (LI-COR). Fluorescent signals (red color for IRDye 800) from spots were evaluated with GenePix Pro4 software (Axon Instruments, DIPSi Industrie, Nanterre, France). The background was subtracted, taking into consideration the signal intensity from spots of nonmimetic peptides carrying random sequences, and then data were transformed into Excel tables or histograms. The SD was calculated by comparison of the fluorescence intensity data from duplicate spots of printed samples in two independent experiments.

ELISA was performed in microtitration wells (Nunc, Roskilde, Denmark) by incubation of the phage suspension (100 µL,  $1 \times 10^{10}$  PFU/mL in BSA/PBS *per well*) overnight at 4°C. Unbound phages were discarded, wells were washed 3 times with PBS/0.3% Tween, then blocked with 1% BSA/PBS for 1 h at 37°C, incubated with IgG (5 µg/well) in PBS/0.3% Tween for 1 h at 37°C and washed with the same buffer. Bound antibodies were detected using anti-human alkaline-phosphatase conjugated antibody diluted 1:1000 as described by the manufacturer (Zymed Laboratories, South San Francisco, CA, USA) at 405 nm absorbance with an automated reader. The SD was calculated from three measurements. In order to compare the efficiency of array- and microplate-based immunoassays, we compared the antibody binding prevalence expressed as a ratio between the fluorescence intensity monitored for the binding to IgG from infected patients *versus* noninfected (negative control) IgG by the protein array method and as a ratio between OD<sub>405</sub> nm values monitored for the binding to the same immunoglobulins by ELISA.

Initially several phage clones were probed with the IgG fraction purified from the serum of patient 1. Equimolar concentrations of clones printed on the same membrane exhibited various signal intensities reflecting their respective antibody binding affinities (see below). However, the fluorescence intensity correlated with the increasing concentration of printed phage particles carrying CXXKXXC mimetic peptides whereas it was negligible for disease-irrelevant phage clones (Fig. 1) indicating that the monitored signal is a function of the quantity of printed capture epitope-specific peptides displayed on the surface of M13 phage particles. Under the experimental conditions used, the fluorescent signal was detectable from spots with nearly  $1 \times 10^6$  immobilized phages exhibiting high



**Figure 1.** Relationship between fluorescence intensity and quantity of printed phage particles. Diluted (2-fold) phage suspensions were printed on a membrane. The membrane was first incubated with 5 µg/mL IgG purified from the serum of patient 1 prior to HAART and then incubated with anti-IgG secondary antibodies labeled with IRDye 800. The curves are obtained from four measurements after subtraction of background fluorescence.

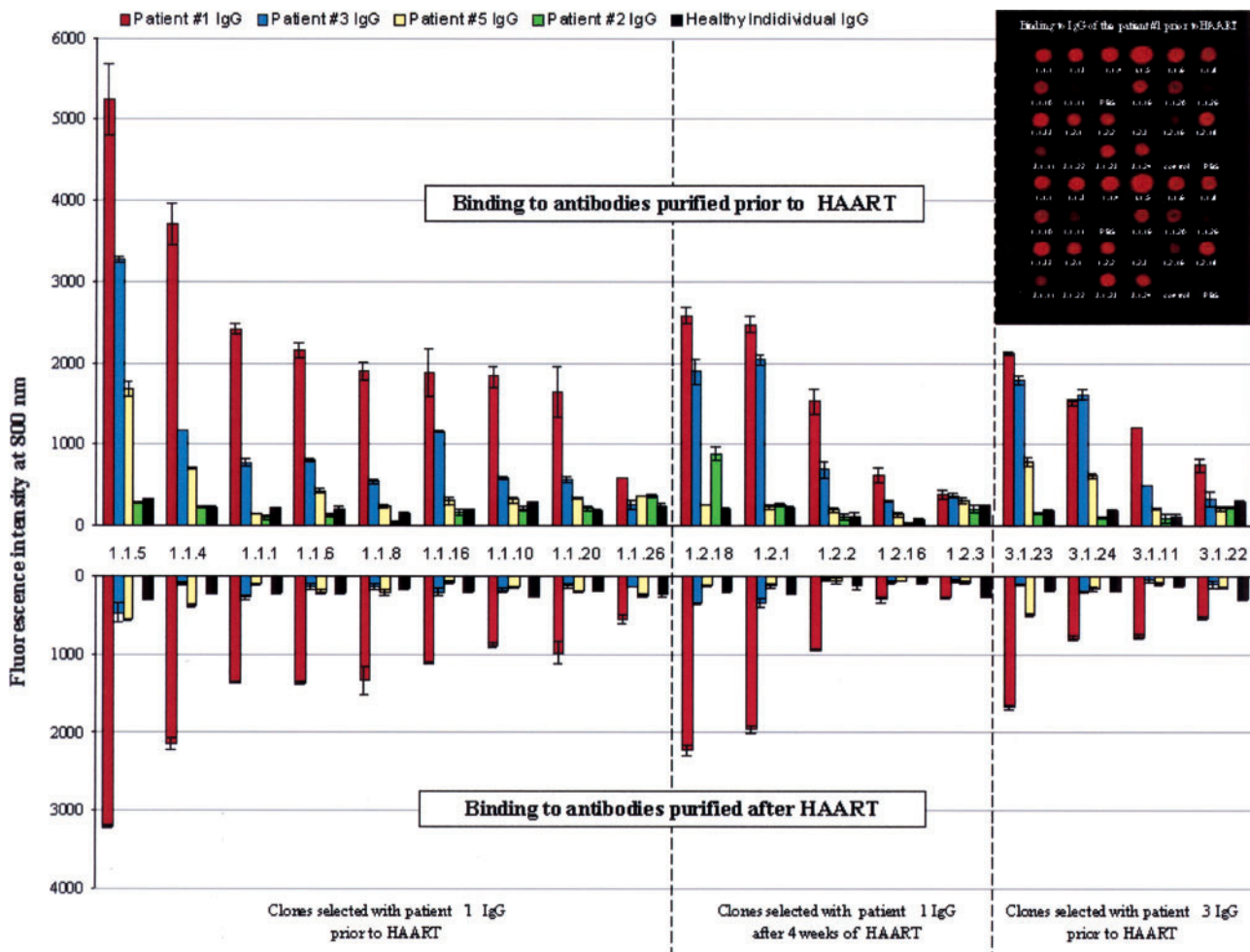
affinity for antibodies. However, the sensitivity of the detection can be increased more than 2-fold on a recently commercialized new formula of NC FAST-slides (Schleicher & Schuell) providing an increased signal-to-noise ratio (data not shown).

To assess the response of the immune system of patients to the HIV-1 virus infection, we fabricated a microarray of 18 phage clones carrying various sequences of the major immunodominant epitope of gp41 (see Table 1)

and tested the immunoassay format in binding to antibodies purified from patients prior to and after the initiation of HAART. All phage clones selected prior to HAART strongly recognized IgG of patients 1 and 3 prior to HAART, except for clone 3.1.22, which had a low affinity for the antibodies used for its selection (Fig. 2, upper part). Only four clones appeared to exhibit a relatively high binding to antibodies from patient 5 as compared with control antibodies. However, no binding could be detected to the IgG fraction of patient 2, which was con-

firmed by ELISA (Gazarian *et al.*, unpublished data) allowing us to assume that the serum of this patient is devoid of the major immunodominant epitope-specific antibodies.

The phage clones selected with the sera of patients 1 and 3 exhibited strong mutual cross-reactivity, except for clone 1.1.26 that bound weakly to the IgG of patient 3. In general, the fluorescent signal was stronger with antibodies from patient 1 than with antibodies from other patients. Clone 1.1.4 also showed higher reactivity with



**Figure 2.** Binding of ordered phage-displayed mimetic peptides to the IgG fraction of sera purified from HIV-1 infected and noninfected individuals. Phage clones 1.1.x and 3.1.x were selected with the sera of patients 1 and 3, respectively prior to HAART; clone 1.2.x was selected with the serum of patient 2 after 4 weeks of HAART. Clones 1.1.3 and 1.1.33 have the same peptide sequences as 1.1.4. Therefore, similar data obtained for these clones are not shown in histograms. The error bars represent the SD of the fluorescence intensity between the printed spots (duplicate spots in two independent experiments). The upper histogram shows the binding efficiency to IgG purified from sera prior to HAART. The lower histogram shows the binding to IgG purified from the sera of patients after HAART (12-week treatment for patients 1 and 5 and a 15-week treatment for patient 3). The IgG fraction of patient 2 was not available after HAART. The IgG of patients are indicated as follows: ■ = IgG of patient 1, ■ = IgG of patient 3, ■ = IgG of patient 5, ■ = IgG of patient 2 and ■ = IgG of the noninfected patient (negative control). An example of the ordered protein array with immobilized phage proteins is shown in the insert (spots are shown in duplicate).

the serum of patient 1 than with the serum of patient 3 by ELISA (data not shown). Furthermore, the cross-reactivity of three out of the four phage clones (except clone 3.1.24) selected with the serum of patient 3 was higher with respect to the antibodies of patient 1 as compared to its own selecting antibodies (see Fig. 2). This indicates that the concentration of HIV-1-specific antibodies against the immunodominant epitope in the four tested sera of patients before HAART was highest in the serum of patient 1. In addition to the cumulative acquisition of mutations, inter-virus genome recombination and the selective pressure originating from the immune system, anti-retroviral drug therapy also contributes to the genetic variability of HIV-1 [see 14 for a recent review] and can cause the replacement of the wild-type virus in plasma by drug-resistant mutants after 2 weeks [15]. With this in mind, we used the same panel of 18 mimotopes to assess the flexibility of the immune response of patients 1, 3 and 5 after the initiation of HAART by using IgG purified from the corresponding sera in the course of the therapy. Fluorescent signals decreased from all spots probed with the antibodies of patients 1, 5 and especially 3 (Fig. 2, downward bars) indicating that the concentration and probably the affinity of HIV-1-specific immunoglobulins directed against the immunodominant epitope significantly decreased.

As judged from the fluorescence intensity of the spots, the phage clones exhibited various affinities for antibodies from patients 1 and 3 prior to HAART (see Fig. 2). The ELISA, used to monitor the antibody-binding strength of 13 mimotopes, supported the accuracy of the array data (see Table 1). These results confirm that substitutions of variable amino acids within the CXXKXXC sequence and adjacent to the motif can dramatically affect the ability of HIV-specific antibodies to recognize the gp41 epitope [16]. However, several phage clones, such as 1.1.1, 1.2.18 and 1.2.1, which were considered strong binders due to their binding to antibodies from patients 1 and 3, exhibited very low affinity for the antibodies of patient 5 prior to HAART. Moreover, only the strongest binder, 1.1.5, exhibited a relatively high binding to the antibodies from patients 3 and 5 after the initiation of HAART. Another clone, 3.1.23, had good affinity for antibodies from patient 5 after HAART whereas all other clones were almost unable to recognize the antibodies of both patients after therapy (see Fig. 2). These observations appear to reflect the polyclonal nature of the antibodies before therapy [13] and the changes in antibody repertoire in patients during therapy [17]. This could lead to a decrease in the level of gp41 immunodominant epitope-specific antibodies in serum as a result of rapid cell turnover and the accumulation of HIV-1 mutants [15] with decreased affinity for initially generated antibodies.

The quantitative measurement and comparative characterization of antibodies elicited by epitopes of viruses causing long-term infections has a prognostic value to evaluate the contribution of the immune system of patients to the defense against the virus. Our data show that array technology, supported by near-infrared detection, can be used as an efficient immunoassay to study antigen-antibody interactions in terms of comparison of mimetic peptide-binding affinity and measurement of the relative concentration of antibodies in the sera of HIV-1 infected patients. With respect to the performance of the experimental method used, the sensitivity of the array method is at least similar to that of ELISA on microplates. Furthermore, the array method requires almost 1000-fold less antigen and 100-fold less antibody samples for assays. In addition, the printing of antigens on membranes provides precise quantification of capture molecules as compared to the coating of antigens on microplate surfaces. Moreover, the antibody-binding prevalence, calculated as the fold binding monitored for the specific-binding to patient IgG over binding to the negative control IgG of a noninfected patient is more remarkable from array measurements than from ELISA (except for clone 1.1.26; see Table 1). Consequently, a phage-displayed mimetic peptide array better reflects the difference between test and control probes and might help to detect sub-optimal antigen-antibody interactions involved in the immune response of HIV-1 infected patients. From the data presented, it becomes apparent that arrays of phage-displayed mimotopes of pathogenic viruses and bacteria can be used to develop suitable immunological tools for the evaluation of the immune repertoires of patients for both prognostic and diagnostic purposes as well as for screening vaccine candidates.

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