Antiretroviral Therapy-Induced Functional Modification of IgG4 and IgM Responses in HIV-1–Infected Individuals Screened by an Allosteric Biosensor

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We have explored the effect of antiretroviral drugs on the antiviral immune response in human immunodeficiency virus-1 (HIV-1)–infected patients by using an enzymatic immunosensor that detects epitope-modifying anti-gp41 antibodies. By this molecular sensing approach, we have identified an irreversible impact of drug administration on the functionality of IgG4 and IgM specific antibodies regarding the structural modification promoted on their target epitope. During the antiretroviral therapy, the prevalent induced fit promoted by IgM on the epitope was lost at the expense of that promoted by IgG4, suggesting alternativeness in the neutralization potency of these antibody subpopulations. Because the particular drug composition of the antiretroviral treatment did not affect such immune shift, the obtained data strongly suggest that the drop in the viral load and the consequent lost of antigenemia are responsible for the functional adaptation observed in the humoral response. (*Journal of Biomolecular Screening* 2008:817-821)

Key words: antiviral drug, immunosensor, HIV-1, humoral response, gp41

INTRODUCTION

THE HUMORAL RESPONSE against the human immunodeficiency virus-1 (hiv-1) infection has long been considered of poor relevance regarding the progression of the infection. However, antibody-mediated viral neutralization and the consequent rising of evasive mutants have been proven as important contributors of HIV-1 dynamics in infected individuals.[1,2] In the case of HIV-1, the neutralization potency of antiviral antibodies directed to *env* gene products (gp41 and gp120) depends on the extent of structural perturbations caused by the antibody binding on the target epitope and surrounding areas.[3,4] This fact might be related to the high conformational flexibility of gp41-gp120 complexes at the virion surface that is required for the structural adaptation of both proteins to the CD4 receptor during cell attachment.[5] The neutralization potency of selected monoclonal antibodies can be predicted through monitoring the enthalpic and entropic changes in the ligand upon antibody binding as a reflection of the extent of induced fit.[3] However, such parameters cannot be obtained from whole sera, and the spectrum of available neutralization assays remains as the only suitable method for evaluation of neutralization capability.[6]

On the other hand, it is known that HIV-1 replication is required for an efficient production and maintenance of significant titers of anti–HIV-1 immunoglobulin (Ig)G antibodies in serum.[7] Consequently, the administration of antiretroviral therapy (ART) has a dramatic, negative impact on the immunoglobulin titer in treated subjects.[7,8] However, there are no data about how ART can modify the functional quality of antiviral antibodies, and how the inhibition of viral replication might affect the neutralization potential and functional distribution among different antiviral antibody subpopulations.

With the aim to determine any eventual functional modification that ART might induce in the antiviral response, we have here examined the capability of different Ig classes to activate

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the allosteric molecular sensor NF795gpC, in sera from both untreated and treated patients.

NF795gpC is an engineered version of the *Escherichia coli* β-galactosidase that displays the main immunodominant epitope from HIV-1 gp41 in a solvent-exposed area of the protein.[9] This hybrid protein responds allosterically to anti-gp41 monoclonal and polyclonal antibodies through conformational perturbations induced by the antibody on the enzyme's active site. Therefore, apart from being a very efficient and sensitive "protein-only" biosensor[9] for the detection of HIV-1 infection,[10] the conformational sensitivity of NF795gpC makes this enzyme a useful tool to evaluate the "induced fit" abilities of specific anti–HIV-1 antibody populations.[11]

MATERIALS AND METHODS

Biosensor description and production

The antibody-sensing β-galactosidase enzyme NF795gpC displays the P1 immunodominant B-cell epitope of HIV-1 gp41 in a solvent-exposed position.[9] In this location, the accommodated viral peptide is accessible and fully immunoreactive. The binding of specific anti-P1 antibodies produces structural changes that result in detectable variations in the enzyme specific activity, easily detectable in a homogeneous assay. The hybrid enzyme NF795gpC becomes then an allosteric nanosensor[10] in which the P1 peptide acts as the specific receptor and antibodies as allosteric effectors. NF795gpC was produced in *E. coli* BL26 strain through the thermosensitive expression vector pNF795gpC. After 4 h of induction at 42 °C, protein was centrifuged and purified by conventional affinity chromatography.[11] The harvested protein was then dialyzed against Z buffer,[12] and it was quantified by Bradford analysis.[13]

Sera samples

A total of 57 HIV-1–infected human sera were used in this study (**Table 1**). Eighteen were from healthy individuals, 20 from HIV-1–infected patients who received ART without interruption for 55 weeks (mean; range 16-120), and 19 from HIV-1–infected patients who underwent structured treatment interruption for 30 weeks (mean; range 2-112 weeks). All the sera were tested for NF795gpC allosteric activation. Sera from 8 noninfected individuals were included in the analysis as negative controls.

Biosensing analysis

The enzymatic sensing assay was performed in 96-well microplates by incubating 20 µl of 1:4 dilution of each serum in Z buffer (pH 7.0; 0.06 M Na_2HPO_4, 0.04 M $NaH_2PO_4H_2O$, 0.01 M KCl, 1 mM MgSO4 $7H_2O$) with 10 µl of 4.16 nM NF795gpC per well. After incubation for 1 h at 25 °C, 40 µl of 1.66 mM chlorophenol red β-D-galactopyranoside (CPRG, a lactose analogue rendering red hydrolysis products) in Z buffer were added. After 30 min of reaction, the absorbance was determined at 540 nm in a Labsystems iEMS plate reader. Percentages of enzyme activation were determined by referring to values obtained in absence of sera and used as a convenient numeric biosensing signal. All the experiments were performed in triplicate.

ELISA

For antibody class and subclass analyses, high binding Costar ELISA plates were coated with saturating concentrations of NF795gpC (7 pmol per well) and 100 µl of sera previously diluted in PBS at 1:500 was added to each well. Different types of anti-human secondary antibodies were used at optimal dilutions (anti-IgG, 1:2000 from Pierce, Rockford, IL; anti-IgG1, 1:500; anti-IgG2, 1:2000; anti-IgG3, 1:1000; anti-IgG4, 1:2000; anti-IgM, 1:2000; anti-IgA, 1:1000, and anti-IgE,1:500 from Southern Biotechnology Associates, Inc., Birmingham, AL). The ELISA reaction was developed with 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) from Sigma (St. Louis, MO) and antibody binding measured at 405 nm. The determinations were performed in a minimum of 14 sera samples from all the cohorts. When the numerical study required more data for significance analysis, the determination of antibody composition was extended to a bigger number of samples. All the experiments were performed in triplicate.

Statistical analysis

Correlation between the activation levels of NF795gpC promoted by each sera sample and titers of the different types of Igs found in the same sera were done using the linear regression analysis tool from SigmaPlot version 10.0. The comparisons between activation levels in sera sets according to Ig titer and group of treatment were performed by the Kruskal-Wallis nonparametric statistical test. *P* values of less than 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

To determine the possible impact of the ART in the humoral response against HIV-1, we determined which specific antibody populations are responsible for the sera-mediated NF795gpC activation in both nontreated and ART-treated HIV-1–infected individuals. For that we explored, in a selected set of sera samples (**Table 1**), the correlation between the sera-mediated activation of NF795gpC (by the increase in the β-galactosidase activity) and the prevalence of different classes of antibodies (by immunoreactivity with the P1 B-cell epitope of HIV-1 displayed by NF795gpC).

Table 1. Clinical Parameters and ART Status of Sera Source Patients

Sera Reference	*Viral Load (HIV-1 RNA Copies)/ml*[b]	*CD4*$^+$ *Cells/μl*[c]	*CD8*$^+$ *Cells/μl*[c]	***ART***[c]
Nontreated				
1	752855	10	150	
2	22500	300	612	
3	195000	616	1380	
4	167000	273	682	
5	28406	1049	935	
6	327165	388	957	
7	34328	273	na	
8	51028	610	1196	
9	20200	752	1040	
10	4270	572	1360	
11	72800	508	1680	
12	1960	584	1540	
13	5440	515	1470	
14	50800	276	959	
15	1520	240	1302	
16	71300	420	800	
17	150000	759	1182	
18	63856	1100	1952	
Treated				
Continuous treatment				
19	467433	10	1708	AZT, 3TC, DDC
20	271648	180	1740	AZT, 3T, DDC
21	41063	285	2345	AZT, DDC
22	9564	350	622	DDI, HU
23	233430	46	472	3TC, D4T, SQV
24	53967	108	720	HU, D4T
25	57	770	1155	D4T, 3TC, IDV
26	<20	635	1271	ABV, 3TC, IDV
27	<20	515	528	ABV, 3TC, IDV
28	<20	884	na	ABV, 3TC, IDV
29	<20	625	878	3TC, NFV, AZT
30	<20	631	711	DDI, 3TC, NVP
31	<20	889	2148	ABV, 3TC, NVP
32	<20	562	777	D4T, IDV, 3TC
33	<20	392	595	DDI, 3TC, NVP
34	<20	388	949	DDI, 3TC, NVP
35	<20	1165	915	D4T, 3TC, NFV
36	<20	944	1285	D4T, 3TC, NFV
37	<20	342	935	D4T, IDV, 3TC
38	<20	1236	1169	DDI, IDV, NFV, NVP
Interrupted treatment				
39	15100	840	1610	DDI, D4T, 3TC
40	58500	352	1230	AZT, 3TC, ABC, EFV
41	113000	493	748	IDV, 3TC, AZT
42	14400	720	881	EFV, D4T, 3TC
43	15500	725	1485	ABV, DDI, LPV, RTV
44	12200	216	798	ABV, DDI, EFV
45	18900	342	1240	ABV, DDI, NVP
46	5130	405	822	EFV, 3TC, AZT
47	19600	270	562	3TC, LPV, RTV, TDF
48	8630	840	865	ABV, 3TC, NVP
49	16700	342	1471	DDI, 3TC, NVP, TDF
50	9050	516	780	D4T, 3TC, NFV
51	6810	425	960	3TC, NFV, AZT
52	18400	1209	1211	ABV, 3TC, NVP
53	11700	902	1476	DDI, D4T, NFV
54	14765	814	910	D4T, 3TC, NFV
55	16100	756	1392	ABV, 3TC, LPV, RTV
56	6560	380	548	DDI, 3TC, LPV, RTV
57	15700	1287	1986	3TC, NFV, AZT

ART, antiretroviral therapy; HIV-1, human immunodeficiency virus-1.
[a]Plasma HIV-1 RNA levels were measured by use of the ultrasensitive Amplicor monitor assay (Roche).
[b]CD4 and CD8 cell count was determined by flow cytometry.
[c]3TC, lamivudine; ABV, abacavir; AZT, zidovudine; D4T, stavudine; DDI, didanosine; EFV, efavirenz; IDV, indinavir; LPV, lopinavir; NFV, nelfinavir; NVP, nevirapine; RTV, ritonavir, PI; TDF, tenofovir; na, data not available.

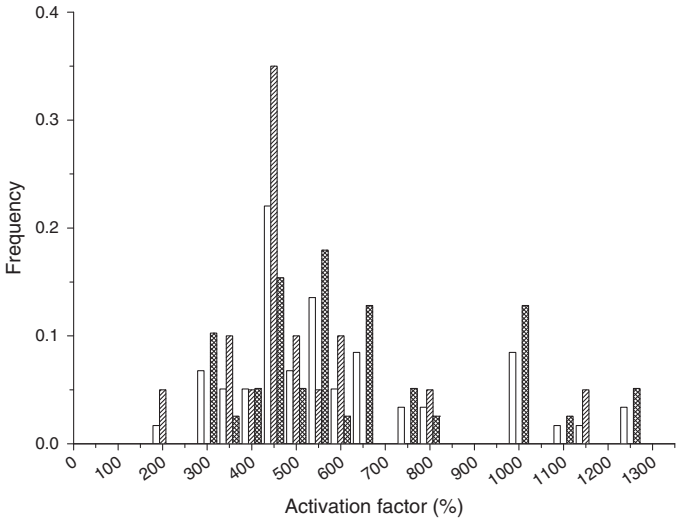

FIG. 1. Distribution of patient frequency regarding sensor activation levels promoted by individual patient sera (increase of enzymatic activity, as %). Patient frequency was calculated by referring the number of patient sera in each group of activation to the total number of patient sera within the group of treatment (total patients, treated and nontreated patients). White bars represent the total number of patients, right-dashed bars the nontreated patients, and rhombus-filled bars the total of patients who had followed an antiretroviral therapy.

Table 2. Significance of Correlations (Given as *P* Values) Between the Sensor Activation and the Prevalence of Ig Types in Each Serum

Antibody	*Nontreated*	*Treated*	*Total*
IgG1	0.159	0.545	0.162
IgG2	0.946	0.811	0.874
IgG3	0.741	0.838	0.800
IgG4	0.128	0.098	0.025*
IgA	0.803	0.528	0.533
IgE	0.099	0.364	0.105
IgM	0.005*	0.671	0.201
VL[a]	0.642	0.388	0.140
CD4	0.506	0.535	0.305
CD8	0.057	0.812	0.592

*Asterisks indicate *P* values below 0.05.
[a]Relevant clinical parameters of patient source of sera have been included as controls.

The profiles of biosensor signals induced by sera (**Fig. 1**) were indistinguishable when comparing sera from ART patients and sera from nontreated patients, as confirmed by a Kruskal-Wallis test ($H = 3.37$, $p = 0.06$). Negative controls, namely 8 sera from noninfected individuals, showed no sensor activation as expected (not shown). When investigating possible linear dependence between activation factors and the amount of specific antibody subpopulations, we found that, as described previously, the levels of allosteric activation of NF795gpC correlated significantly ($P = 0.025$) with the prevalence of anti–HIV-1 IgG4 in sera (**Table 2** and **Fig. 2B**). This result indicates a main contribution of such IgG subclass in sensor activation. However, when analyzing exclusively the sera set from nontreated individuals, the dependence between the sensor activation signals and IgG4 titers was not observed. Instead, in absence of antiviral treatment, the occurrence of specific anti-gp41 IgM antibodies clearly determined the extent of NF795gpC activation ($P = 0.005$; **Fig. 2A**).

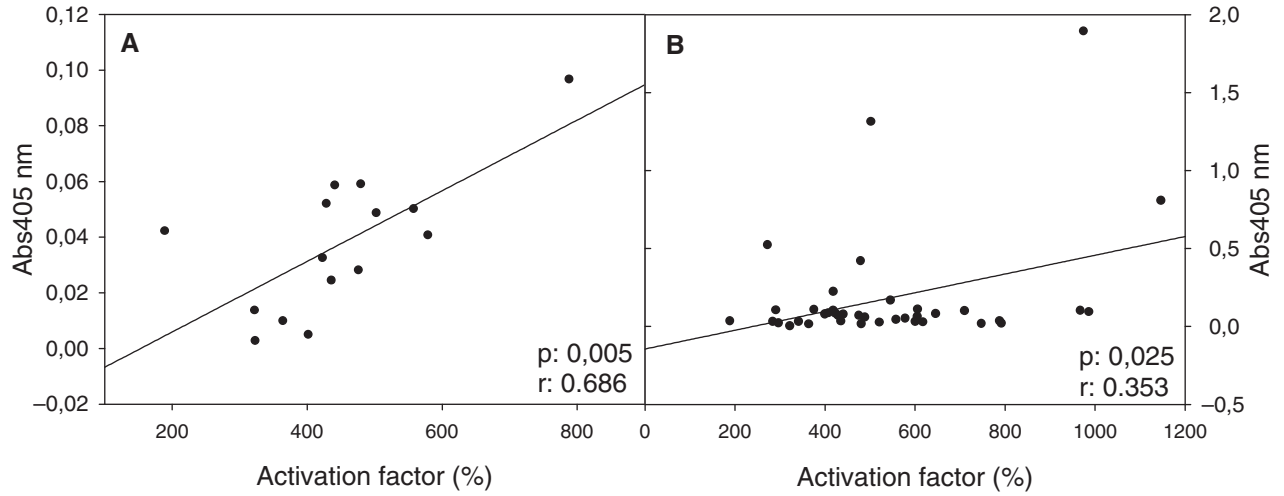

FIG. 2. Scatter plots and correlation values between activation of the sensor (represented as a percentage of activation, %) and IgM (A, for nontreated patient sera) and IgG4 (B, for total patient sera) concentration (given as A_{405}). Correlation parameters were obtained through the linear regression analysis tool from SigmaPlot version 10.0.

Because the composition of the ART was nonhomogeneous (**Table 1**), the shift in the contribution of specific immunoglobulin populations to the sensor activation could not be attributed to any specific drug. In addition, ART intervals were also diverse in the set of analyzed individuals. In those in which the treatment had been interrupted, viral load values were notably high, indicating the recovery of viral replication after interruption (**Table 1**). Because both sera populations (from patients receiving interrupted ART and continuous treatment) were statistically indistinguishable regarding sensor activation ($P = 0.48$; not shown), the changes in the functionality of the antiviral response caused by ART are not reverted by a late recovery of virus multiplication, and are, therefore, irreversible.

In summary, in naïve HIV-1–infected individuals, IgM is the antibody class most efficiently modifying the immunodominant B-cell epitope of gp41, but in individuals receiving ART there is a clear functional dominance of IgG4. Such effect is irrespective of both the specific drugs used in ART and the continuity of the treatment, and therefore, it does not depend on low viral replication or load. Therefore, the drop of antigenemia generally associated with ART is probably causing a functional adaptation of the humoral response in which IgG4 gains neutralization competence at the expense of IgM.

Importantly, the results shown here stress the value of allosteric nanosensors as powerful tools for functional and dynamic screening of individual anti-HIV immune responses, beyond their utility as mere diagnostic tools.

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