New Method for Quantifying Anti-HIV1-gp160 Antibodies in Saliva, Cervicovaginal Secretions, and Serum of Infected Women

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Antibody titers or units as measured by ELISA or similar assays represent only a semi-quantitative measurement of the antibody concentration. In the present study, a truly quantitative assay for evaluating antibody concentrations was developed. In the new assay, IgG and IgA antibody concentrations are expressed in absolute gravimetric units (weight/ml) rather than by more ambiguous terms such as titers or units. The procedure is based on the comparison of known concentrations of IgG or IgA bound to anti-F(ab)₂ with the binding of an unknown concentration of antibodies to

a specific antigen. We applied the new assay to the measurement of HIV1-gp160 specific IgG and IgA concentrations in cervicovaginal secretions (CVS), saliva, and sera of infected women. Measurement of antibody activity was obtained by comparing the amount of specific antibodies to the amount of total immunoglobulins found in the same compartment. Because of its quantitative and comparative nature, the assay was named comparative antibody gravimetric evaluation assay (CAGE). J. Clin. Lab. Anal. 14:188–192, 2000. © 2000 Wiley-Liss, Inc.

Key words: HIV1-gp160; antibody activities; ELISA

INTRODUCTION

The enzyme-linked immunosorbent assay (ELISA) and radioimmunoassays (RIA) are widely accepted procedures for approximating antibody or antigen levels. The above methods are based on using labeled antibodies as detection reagents (1,2). When specific antibodies are detected using the indirect ELISA assay, the results are presented as either ratios of Δ OD/cut-off values, titers, or ELISA units. All these antibody measurements are semi-quantitative. Furthermore, assignment of antibody activity by ELISA units can be misleading when EU/ml involves more than one isotype. To calculate the cut-off value, a large number of normal control sample are needed to achieve statistical significance. To obtain detection limit titers, some samples must be highly diluted to reach Δ OD value less than 0.1; therefore, extra pipetting may create additional errors (3). In addition, a large quantity of antigen for coating the plates is needed. Antibody concentration is more accurate by absolute gravimetric units (weight/ml) than by semi-quantitation units, or titers.

Generally, in order to measure antibody concentration in a sample, the antibody concentration is extrapolated from a standard curve generated from data based on a known concentration of antibody solution. Antibody standard solutions can be obtained using chromatography and immunosorbent procedures. However, this purification of antibodies is cumbersome

and time consuming. The concentration of purified antibody in a standard solution is then determined by spectrophotometric (A280) or colorimetric methods using bovine serum albumin as the standard protein. Considering the difficulties in establishing a known concentration of standard HIV-gp160 antibody solution, we developed and standardized a technique to evaluate the HIV-gp160 antibody concentrations in absolute quantitative gravimetric units (weight/ml) without having to use the complex standard antibody solution. We tested the accuracy of the new assay by repetitive measurement of antibody concentration in pooled CVS, saliva, and sera obtained from infected women.

MATERIALS AND METHODS

Samples

Saliva, CVS, and serum were collected from eight women infected with HIV-1 (CDC II–IV). All patients had serum anti-HIV-1 antibodies as determined by ELISA (Abbott Laboratories, third generation, Abbott Park, IL, and ELAVIA sanofi, Diagnostic Pasteur, Marnes-la-coquette, France) and con-

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firmed by Western blot (Abbott Laboratories). The samples were aliquoted and stored at -80°C until use. Serum, saliva, and CVS samples were pooled prior to testing.

Collection of Saliva and Cervicovaginal Secretions

Whole saliva was collected by inserting a sterile cotton pad into the mouth under the tongue (OMNI-SAL, SDS, Vancouver) for more than three minutes; the detailed collection procedure was previously described (4). The saliva samples were filtered, aliquoted, and immediately frozen at -80°C within 2 hours. The CVS samples were collected as previously described (5).

Immunochemical Reagents

Human immunoglobulins

Human IgG and sIgA were purified as previously described (6). Briefly, human IgG from pooled human sera was purified by a combination of neutral salt precipitation (47% w/v) and DE-cellulose 52 (Whatman, England) anion-exchange chromatography. Secretory IgA (sIgA) from human colostrum was purified by gel filtration on Sepharose 4B, DEAE-cellulose column and affinity chromatography. Each immunoglobulin isotype preparation was free of other isotypes as assayed by immunodiffusion and by indirect ELISA following coating of $2\text{ }\mu\text{g/ml}$ of IgG or IgA on the solid phase. The purified IgG and sIgA were also used as antigens to immunize goats for obtaining the specific antisera.

Antisera to human immunoglobulins

Anti- γ (goat) and anti- α (goat) were purified by passage of the immune sera on the corresponding immnosorbent column, and polyclonal IgG anti-sera was peroxidase-labeled by the Nakane method (7).

Ratio-iodined human immunoglobulins

The purified human IgG was pooled from more than 150 individuals, and purified sIgA was pooled from the colostrum of more than 60 women. Purified IgG and sIgA were iodinated by a standard iodination procedure (8). The iodinated immunoglobulins were used as standards for quantitation of immunoglobulin isotype. The radioactivity of the labeled IgG and sIgA were measured by LKB γ -counter and were determined as 4,500 CPM/ng for labeled IgG, and 3,200 CPM/ng for labeled sIgA.

Preparation of IgG goat anti-F(ab) $'_2$ human

IgG goat anti-F(ab) $'_2$ was prepared by the method described elsewhere (9). The solution of human IgG and Pepsin (Calbiochem, La Jolla, CA) was crystallized three times and

then dissociated in 0.2 M sodium acetate buffer (pH 4). Pepsin and IgG were mixed at a ratio equal to 1:50 (V/V) for 24 hours at 37°C without agitation. The reaction was stopped by neutralization with 1 M phosphate dipotassic buffer pH 8.3. The F(ab) $'_2$ was purified on a column of Sephadex G 100, $2.5 \times 100\text{ cm}$, and equilibrated with PBS containing 1% sodium azide. By this procedure, the IgG F(ab) $'_2$, and Pepsin were separated. The F(ab) $'_2$ was used as immunizing antigens to prepare antisera. The IgG goat anti-F(ab) $'_2$ was purified by the DEAE-cellulose 52 (Whatman, England) anion-exchange chromatography methods.

HIV1-gp160 LAI

HIV1-gp160 was obtained from a Transgene Laboratory S.A (gp160 LAI, protein recombinant, Molsheim Strasbourg, France). The optimal concentration of HIV1-gp160 ($5\text{ }\mu\text{g/ml}$ in PBS) for coating wells was previously described (5).

Quantitation of Anti-HIV1-gp160 Antibodies by the New Comparative Antibody Gravimetric (weight/ml) Evaluation Assay (CAGE)

Immuno 2 removewells (Dynatech, Switzerland) were coated with goat IgG anti-F(ab) $'_2$ ($4\text{ }\mu\text{g/ml}$, $100\text{ }\mu\text{l/well}$ in PBS at pH 7.2, 150 mmol/L) overnight at 4°C . The plates were then washed two times with PBS containing 0.1% Tween 20 and blocked with skim milk at 4% in PBS for 3 hours at 37°C . After removing the blocking solution, the 125-I-IgG or 125-I-sIgA was added in sequential dilutions ($1,000\text{ ng/ml}$ – 0.97 ng/ml) to the plate, and incubated for 1.5 hours at 37°C , and then washed 3 times with PBS-Tween 20. The radioactivity (CPM) of each well was measured by a LKB γ -counter. After the CPM counting, the microwells were remounted on the plate and incubated with peroxidase-conjugated goat anti-human Fc(α) ($2\text{ }\mu\text{g/ml}$, $100\text{ }\mu\text{l/well}$), or goat anti-human Fc(α) ($2\text{ }\mu\text{g/ml}$, $100\text{ }\mu\text{l/well}$) for 1 hour at 37°C . The microwells were then washed 3 times with PBS-Tween 20 buffer. The plate was then developed, with orthophenylenediamine (OPD) as the substrate, for 5 min. The reaction was stopped by H_2SO_4 4N and the OD values were measured by spectrometer at 492 nm, reference 620 nm. Because of the double labeling, each well was measured by both γ -counter and spectrometer. The ng/ml of 125-I-IgG or 125-I-sIgA bound was determined based on radioactivity (CPM), so that the relationship between substrate conversion in the linear region of the logarithmic titration plot and the ng/ml of IgG or sIgA bound in exactly the same wells was established.

The microwells coated with HIV-1gp160 proceeded along the same blocking and washing procedure in the same plate. Serum was serially diluted from 1/64,000 to 1/512,000 for IgG and from 1/40 to 1/320 for IgA. Saliva was serially diluted from 1/10 to 1/80 for IgG and from 1/5 to 1/40 for IgA. CVS was serially diluted from 1/100 to 1/400 for IgG and from 1/20 to 1/160 for IgA. Samples were incubated in wells

for 1.5 hours at 37°C and peroxidase conjugated anti-human antibody was applied. The wells were developed in the same way as the anti-F(ab)₂ coated wells. Therefore, the concentration of HIV1-gp160 antibodies was evaluated by comparing known concentrations of IgG or IgA binding to anti-F(ab)₂ with an unknown concentration of antibody binding to specific antigen in the same plate.

To insure that all the antibodies were bound and measured, the sample from each well after the first incubation was transferred to a new HIV1-gp160 and IgG goat anti-F(ab)₂ coated plate. The detection procedure was the same as described above. The above transfer was repeated two times to insure the absorption of all the antibodies in each well. A Δ OD of less than 0.1 was considered as the end point at which no antibody remained in the diluted samples. The Δ OD was determined by subtracting the OD values in the control wells from the OD values in the test wells. The total antibody concentration was computed by adding the antibody concentrations obtained in the first, second, and third absorption.

Measurement of Total IgA, IgG in Saliva, Cervicovaginal Secretions, and Serum

The measurement of total immunoglobulins G and A in saliva and CVS was done as previously described (4). Serum IgG and IgA concentrations were determined by the nephelometric method (Behringwerke AG, Marburg, Germany) (4).

Statistical Analysis

Statistical analysis was done using Anova unpaired Student's *t*-test for comparison of the difference between the antibody activity in CVS, saliva, and sera. The *P* value of < 0.05 was considered as significant difference.

RESULTS

Reproducibility of the CAGE Assay

In order to determine the reproducibility of the CAGE assay for quantitating IgA and IgG antibodies, the intra-assay and inter-assay coefficients of variation were tested. The CPM intra-assay coefficient of variation was 7.9 % for IgG antibody and 9.9% for IgA antibody, while the OD value intra-

TABLE 1. Characterization of the comparative antibody gravimetric evaluation (CAGE) assay

Characteristic	IgG	IgA
Intra-assay coefficient of variation (%) [*]		
CPM / OD	7.9 / 6.3	9.9 / 6.9
Inter-assay coefficient of variation (%) ⁺		
CPM / OD	9.5 / 9.1	13 / 9.2
50% binding of linear standard curve (ng / ml) [§]	8	4
Detection limit (ng / ml) ⁺⁺	1	0.1

^{*}10 samples tested in eight triplicate wells in the same plate.

⁺One sample assayed in six different tests.

[§]Medians of the values from at least ten different inter-assay tests.

⁺⁺Mean + 3 s.d. of the background OD values from at least 10 ELISA plates.

assay coefficient of variation was 6.3 % for IgG antibody and 6.9% for IgA antibody. The inter-assay coefficient of variations for IgG and IgA antibody were less than 14% (Table 1).

Sensitivity of the CAGE Assay

The sensitivity of the CAGE assay was found to be about 1 ng/ml for detection of IgG antibody and 0.1 ng/ml for IgA antibody. The cut-off value was calculated as the mean of blanks + 3 S.D. in which the sample was replaced with 4% skim milk in PBS. The values of 50% binding of the linear part of standard curve were 8 ng/ml for IgG antibody, and 4 ng/ml for IgA antibody (Table 1).

Evaluation of HIV1-gp160-Specific Antibody Activities by CAGE Assay

After transfer of the samples by the CAGE method, the total concentrations of anti-HIV1-gp160 antibodies IgA and IgG were obtained according to the standard curve of 125-I-IgA and 125-I-IgG (Table 2; Fig. 1a,b). The levels of total IgG and IgA in serum were tested by nephelometry, while IgG and IgA in CVS and saliva were tested by sandwich ELISA (Table 2). The antigen-specific antibody activity in serum was, as expected, relatively lower than in other tissues due to the high concentration of total IgG and IgA in sera.

TABLE 2. Evaluation of anti-HIV1-gp160 antibodies by comparative antibody gravimetric evaluation (CAGE) assay and measurement of total IgA and IgG in saliva, cervicovaginal secretions, and serum of infected women

Sample (pool)	<i>n</i>	IgA anti-gp160 ¹	IgA ¹	IgA anti-gp160 activity ²	IgG anti-gp160 ¹	IgG ¹	IgG anti-gp160 activity ²
Serum	8	1.4 ± 0.5	3800 ± 150	0.04 ± 0.001	570 ± 50	22000 ± 100	2.59 ± 0.2
CVS	8	15 ± 5	99 ± 6	15.15 ± 4.0	35 ± 10	252 ± 15	13.88 ± 3.0
Saliva	8	0.3 ± 0.1	82 ± 18	0.37 ± 0.02	0.8 ± 0.2	4.8 ± 0.3	16.67 ± 5.0

Results were given as mean plus standard deviation.

CVS: Cervicovaginal secretions.

¹μg/ml.

²The concentration of antigen-specific antibody / the concentration of total immunoglobulin × 100.

Fig. 1. Comparative antibodies gravimetric evaluation sIgA and IgG assay. (a) sIgA concentration calculated based on 125-I-sIgA of serial dilutions captured on the goat anti-F(ab)₂-coated plate. The 125-I-sIgA that was serially diluted from 1 ng/ml to 2,000 ng/ml was added, in duplicate, to the wells. The bound 125-I-sIgA was calculated based on the CPM/min value of each well. The standard curve was generated after the plates were developed as described in Materials and Methods. The limit of de-

tection was defined as the minimum concentration of sIgA just above the cut-off value. The cut-off value was determined as the mean OD value of the sIgA omitted wells + 3 S.D. Based on this definition, 10 pg of sIgA per 100 μ l of sample was the detection limit. (b) IgG standard curve was generated the same as for the sIgA described above. One hundred pg of IgG per 100 μ l of sample was the detection limit. The correlation coefficient values (r) were demonstrated.

The IgG antibody activity in sera was significantly higher than IgA ($P < 0.01$). The IgA and IgG antibody in CVS exhibit about equal activity (the mean value is 14%). Similar to the serum, IgG antibody activity in saliva was significantly higher than IgA ($P < 0.01$) due to a higher concentration of total IgA in saliva. The highest IgA antibody activity was found in CVS. The IgG antibody activities in CVS and saliva were at similar levels and both were higher than that of sera.

Correlation of the CAGE Assay With ELISA

Validation and correlation of the new CAGE assay with previously established indirect ELISA was accomplished by comparing the results of CAGE assay to those of ELISA run in parallel on the same samples. The higher levels of the Δ OD/

CO ratio always correspond to a higher concentration of antibody (μ g/ml) as measured by the CAGE assay (Table 3).

DISCUSSION

Specific antibody titers or units as obtained by ELISA and similar serological assays represent a semi-quantitative mean to evaluate the presence or increase in antibody concentration following onset of infection or vaccination. In order to translate titers to real quantitative values (weight/ml), one must include standard antibody calibrators in every assay. It is difficult to generate the standard antibody calibrators considering the large variety of possible antigens to be tested. One example of the cumbersome procedure for quantitating anti-SIV p27 antibody is described by Lehner et al. (10). In this

TABLE 3. Correlation of the CAGE method to indirect ELISA for measuring anti-gp160 IgG and IgA antibody in sera, CVS, and saliva

Sample	<i>n</i>	CAGE assay ¹ (μ g/ml)		Indirect ELISA ² (Δ OD/CO)	
		IgA anti-gp160	IgG anti-gp160	IgA anti-gp160	IgG anti-gp160
Sera	8	1.4 $\pm$ 0.5	570 $\pm$ 50	4.5 (3.7–5.2)	18.6 (17–19)
CVS	8	15 $\pm$ 5	35 $\pm$ 10	3.9 (2.9–4.9)	19 (15–22)
Saliva	8	0.3 $\pm$ 0.1	0.8 $\pm$ 0.2	2.1 (0.8–4.8)	18 (11–65)

¹Results were expressed as mean plus standard deviation in μ g/ml.

²Results were expressed as mean plus 25th–75th percentiles. Serum was diluted 1/1000 for IgG antibody, 1/100 for IgA antibody. CVS was diluted 1/10 for IgG antibody, 1/5 for IgA antibody. Saliva was diluted 1/10 for both IgG and IgA antibodies. Cut off (CO) was determined as the mean Δ OD value plus 3 $\times$ s.d. from 15 normal subjects.

procedure, in order to establish IgG and IgA anti-SIV p27 standards, IgG was separated from serum by ion-exchange chromatography on a DEAE-cellulose column and IgA was separated from serum by gel-filtration chromatography on a Sephadex G-200 column. Purified IgG and IgA was then applied to a Sepharose 4B column with bound p27 (10). Another procedure to quantitate antibody concentration is the sandwich ELISA, which was used to evaluate the concentration of human IgM and IgG anti-lipopolysaccharide (anti-LPS) antibodies (11). The above study used goat anti-human Fc(γ or μ) to bind human IgG and IgM while comparing these with human antibodies bound to LPS. Because the site of binding to the human antibodies is different in both systems, an accurate quantitation is questionable. The assay developed in the present work overcomes the above problems by comparing the binding of known quantities of human antibodies to goat antihuman F(ab) $'_2$ with the binding of unknown quantities of the antibodies to HIV1-gp160. In both cases, the ELISA detection antibody is bound to the same Fc site. It has been established that optimal iodination of immunoglobulins does not change their tertiary structure or the affinity and avidity of binding by anti-Fc antibodies (8). We have previously compared the sensitivity of protein A binding to IgG Fc and goat anti-F(ab) $'_2$ binding to IgG F(ab) $'_2$ for measuring human IgG. IgG binding to protein A is 30 times less sensitive than binding to anti-F(ab) $'_2$ (data not shown). The comparative antibody gravimetric (weight/ml) evaluation assay developed here is more sensitive than a similar assay developed by Butler et al. (12) that used goat anti- κ and anti- λ as capture antibodies. In our assay, we used purified IgG goat anti-F(ab) $'_2$ which exhibited a much higher affinity and sensitivity than do IgG goat anti- κ and anti- λ light chains, for capture of either IgG or sIgA (data not shown).

It is better to pre-titrate the sample prior to running the CAGE assay. The titration data allow the start of the CAGE assay with a proper dilution of a sample so that maximum antibodies will be captured in the first run of the test ($\Delta OD < 0.1$) without having to do a second or third transfer.

One of the benefits of the new quantitative procedure (CAGE) is the ability to compare the amount of specific antibodies present in CVS, saliva, or serum with the total amount of the immunoglobulins present in the same compartment. The above data can thus be expressed as a percentage of antibody activity value denoting the percentage of antibody in the total immunoglobulin isotype. The CAGE assay can be applied to quantitate antibodies to any kind of

antigens without having to use a corresponding antibody standard in each test. This method may be particularly adapted to quantitate and compare isotype antibody activity in the different tissue compartments.

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REFERENCES

1. Engvall E, Perlmann P. Enzyme-linked immunosorbent assay (ELISA), quantitative assay for immunoglobulin G. *Immunochemistry* 1971;8:871-874.
2. Mishell BB, Shiigi SM, editors. Selected methods in cellular immunology, solid-phase radioimmune assays. New York: W. H. Freeman; 1980. p 373-379.
3. Lü X, Miller CJ. Concentration of IgG in the sera of normal rhesus macaques as determined by a species-specific radial immunodiffusion assay. *J Immunol Methods* 1996; 197:193-196.
4. Lü X, Delfraissy F, Grangeot-keros L, Rannou MT, Pillot J. Rapid and constant detection of HIV antibody response in saliva of HIV infected patients: selective distribution of anti-HIV activity in the IgG isotype. *Res In Virol* 1994;145(6):369-377.
5. Lü X, Bélec L, Pillot J. Anti-gp160 IgG and IgA antibodies associated with a large increase in total IgG in cervicovaginal secretions from human immunodeficiency virus type 1-infected women. *J Infect Dis* 1993;167:1189-1192.
6. Bouige P, Iscaki S, Pillot J. Immune complexes as immunizing agents to increase the number of monoclonal antibody producing hybrid and to deviate the response to poorly immunogenic epitopes. *Hybridoma* 1990;9:519-526.
7. Nakane PK, Kawaoi A. Peroxidase labeled antibody, a new method of conjugation. *J Histochem Cytochem* 1974;22:1084-1091.
8. Markwell MA. A new solid-state reagent to iodinate proteins, I. Conditions for the efficient labeling of antiserum. *Anal Biochem* 1982; 125:427-432.
9. Lamoyi E, Nisonoff A. Preparation of F(ab) $'_2$ fragments from mouse IgG of various subclasses. *J Immunol Methods* 1983;56:235-243.
10. Lehner T, Bergmeier C, Panagiotidi C, et al. Induction of mucosal and systemic immunity to a recombinant simian immunodeficiency viral protein. *Science* 1992;258:1365-1369.
11. Fomsgaard A, Dinesen B. ELISA for human IgG and IgM anti-lipopolysaccharide antibodies with indirect standardization. *J Immunoassay* 1987;8:333-350.
12. Butler JE, Spradling JE, Petermen JH, et al. Humoral immunity in root caries in an elderly population. I. *Oral Microbiol Immunol* 1990;5:98-107.