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Proof of Principle: An HIV P24 Microsphere Immunoassay with Potential Application to HIV Clinical Diagnosis

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The measurement of CD4 counts and viral loads on a single instrument such as an affordable flow cytometer could considerably reduce the cost related to the follow-up of antiretroviral therapy in resource-poor settings. The aim of this study was to assess whether the HIV-1 p24 antigen could be measured using a microsphere-based flow cytometric (FC) assay and the experimental conditions necessary for processing plasma samples. A commercial anti-p24 antibody pair from Biomaric was used to develop a p24 microsphere immunoassay (MIA) using HIV culture supernatant as the source of antigen. The ultrasensitive Perkin Elmer enzyme immunoassay (EIA) served as a reference assay. Quantification of HIV p24 using the heat-mediated immune complex disruption format described for plasma samples was feasible using the Biomaric MIA and applicable to a broad range of HIV-1 Group M subtypes. The inclusion of a tyramide amplification step was successful and increased the fluorescence signal up to 3 logs as compared with the MIA without amplification. The analytical sensitivity of this ultrasensitive Biomaric assay reached 1 pg/mL, whereas the ultrasensitive Perkin Elmer EIA was sensitive to less than 0.17 pg/mL. Our data indicate, for the first time, that the principle of p24 detection using the heat-denatured ultrasensitive format can be applied to FC. © 2008 Clinical Cytometry Society

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More affordable laboratory monitoring tools are needed for better management of anti-HIV therapy in resource-limited settings. Rather than developing affordable techniques to monitor CD4 counts and HIV viral load (VL) separately (reviewed in 1), it would be simpler and cheaper to combine both assays into a unique flow cytometry instrument. This may result in major advantages in terms of saving laboratory equipment, personal training, time, cost per test, and eventually treatment effectiveness. With the recent commercialization of a new generation of affordable and mobile flow cytometry instruments (reviewed in 2), flow cytometry-based immunoassays have become less expensive and more flexible tools. Assays that measure VL could be adapted to flow cytometry to facilitate follow-up of antiretroviral therapy (ART) in developing countries (3,4). As a consequence, CD4 counts and VL could be measured on the same instrument.

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Several reports have confirmed the utility of the ultrasensitive HIV p24 antigen assay from Perkin Elmer as a surrogate marker for HIV viral load (5–7) and for the monitoring of treatment efficiency. The test is a sandwich enzyme-linked immunosorbent assay (ELISA) with enhanced sensitivity, which is based on heat-disruption of immune complexes and the use of a tyramide-based amplification system (8).

Here, we describe the use of a commercially available p24 antibody combination from the firm Biomaric (Gent, Belgium) to demonstrate that the principle of the heat-denatured p24 antigen enzyme immunoassay (EIA) can be applied to flow cytometry (FC). We have used microbeads as the solid phase and virus supernatant as the source of antigen. The sensitivity of the Biomaric microsphere immunoassay (Biomaric MIA) was evaluated on a panel of HIV-1 primary isolates, derived from several clades using the ultrasensitive Perkin Elmer ELISA (ultrasensitive PerkinElmer EIA) as the reference assay. Finally, the possibility of including a tyramide-amplification step to the Biomaric MIA was examined.

MATERIALS AND METHODS

HIV-1 Isolates

The same stock of IIIB culture supernatants was used throughout the Biomaric MIA development procedure. A panel of 21 HIV primary isolates were derived from 10 different HIV-1 clades (subtype A, B, D, F, G, H, J, A/E, and A/G and Group O) and selected on the basis of the availability of their gag subtype information to evaluate the sensitivity of the Biomaric antibodies towards different HIV-1 subtypes.

P24 Antigen EIAs

The EIA format was used as a reference technique for the development of the MIA. To reproduce the experimental conditions of the heat-mediated immune complex disruption of plasma samples described by Schuppach et al. (8), the EIA from Biomaric and Perkin Elmer were both performed in combination with a tyramide amplification step (ELAST ELISA amplification system, Perkin Elmer). In addition, the HIV samples were all diluted 1 to 6 in phosphate buffered saline (PBS) + 0.5% Triton X and heated for 5 min at 100°C as previously described (8). Since HIV culture supernatants do not contain anti-p24 antibodies, no immune complex disruption buffer was used. The two EIAs were thus referred to as ultrasensitive Biomaric and Perkin Elmer EIA, respectively, and were used in parallel with the MIA to analyze HIV culture supernatants.

Ultrasensitive p24 Perkin Elmer EIA. This served as a reference assay and was performed as previously described (8). The background reactivity cut-off was determined by calculating the average absorbance of three wells containing PBS + 0.5% Triton X-100 plus three times the standard deviation. The absorbance values were read at 450nm with the reference at 630 nm using an endpoint reading protocol with a BIO-RAD 550 microplate reader. The data were analyzed with the microplate Manager 4.0 software (Bio-Rad Laboratories, Hercules).

Ultrasensitive p24 biomaric EIA. Human capture (clone A3CI0E6) and biotinylated human detection antibodies (clone HuMaBio037) were purchased from Biomaric (Gent, Belgium). Ninety-six-well ELISA plates (NUNC, Denmark) were coated with 200 μL/well of the Biomaric detection antibody diluted to a final concentration of 2.5 μg/mL. Remaining binding sites were blocked with PBS + 0.1% casein, washed, and dried in the open as previously described (9). Dry plates were sealed and stored at −20°C until needed. The washing procedure, incubation time, and substrate used for the ultrasensitive Perkin Elmer EIA (8) were also used in the Biomaric EIA to standardize the procedure.

P24 Biomaric MIA

Coating procedure. Covalent coupling of the Biomaric capture antibody was done with the PolyLink coupling kit on 5.61 μm carboxylated beads (both from Bangs laboratories Inc, Fishers, IN, USA), according to the manufacturer's instructions. An extra blocking step of the remaining binding sites with PBS + 1% BSA was added to the coupling procedure. The coated beads were stored at 4°C at a concentration of 10^7 beads/mL in blocking buffer until further use or for a maximum of 1 month. The coating efficiency was verified by staining 0.5 μL of coated beads with 5 μL of anti-human IgG-FITC and analyzed by flow cytometry. The analysis showed a separation of 3 logs of median fluorescence intensity (MFI) between the coated and non-coated beads with no evidence of a loss of coating after 1 month (data not shown).

MIA procedure. Dilutions of the same stock of IIIB were used to demonstrate the proof of principle of p24 detection by FC and to select the appropriate experimental conditions. Subsequently, the Biomaric MIA was evaluated for its sensitivity toward p24 from different HIV-1 clades using the EIA format as a reference. Samples were all heat-treated so that both the MIA and the EIA format quantified the denatured form of p24. In contrast to the EIA format, however, the samples were not diluted 1 to 6 and no tyramide amplification step was used with the MIA at this stage. All the reactions were performed at 37°C in a 250 μL volume. Ten thousand beads were added to the heat-treated undiluted sample and incubated for 1 hour. After a washing step, 1 μg of the paired biotinylated detection antibody was added to the beads and incubated for 1 hour. After a washing step, 1 μL of 1:300 streptavidin-phycoerythrin (PE, Biosource) was mixed with the beads for 5 min at room temperature. The beads were washed with 2 mL of PBS and then resuspended in 200 μL of PBS before FC analysis (FACSCalibur, Becton Dickinson). The instrument was calibrated and standardized every day using fluorescent beads (CaliBRITE, Becton Dickinson) and Facscomp software. The linearity of fluorescent quantitation was

checked weekly using Dako FluoroSpheres (Dako, Copenhagen, Denmark). The analysis of MFI was done at an excitation wavelength of 488 nm on approximately 8,000 beads. The background reactivity cut-off was determined by calculating the average MFI of three samples containing PBS +0.5% Triton X-100 plus three times the standard deviation. Values of MFI obtained from the flow cytometry analysis were entered manually into a microplate manager template file and analyzed with the Microplate Manager 4.0 software (Bio-Rad Laboratories) using the end point protocol. The results were compared with those of the ultrasensitive p24 Biomaric and Perkin Elmer EIA.

Ultrasensitive p24 biomaric MIA. The feasibility of the bead-based assay using the full procedure of heat-mediated immune complex disruption described by Schupbach et al. (8) was tested. The set up included a tyramide amplification step to the MIA protocol in order to improve the lower detection limit of the Biomaric MIA. IIIB virus supernatant diluted 1 to 10 was used as the source of antigen, and PBS + 0.5% Triton-X was used as the negative control. A tyramide directly linked to Oregon green and a tyramide linked to a 14 atom spacer and biotin (tyramide-XX-biotin, Molecular Probes) were compared. The beads were analyzed at an excitation wavelength of 500 nm with tyramide-Oregon green or 488 nm with streptavidin-PE combined with tyramide-XX–biotin. Data were analyzed as described for Biomaric MIA.

P24 Standard Curves

The same batch of series of standards from Perkin Elmer was diluted 1 to 6 in PBS + 0.5% Triton-X, heat-denatured and tested in duplicate with the MIAs and EIAs to generate standard curves. The analytical sensitivity or lower limit of detection was defined as the average of the two lowest p24 concentrations giving a signal change on the standard curve. The background reactivity was defined as the average signal of three samples containing PBS + 0.5% Triton X-100 plus three times the standard deviation.

RESULTS

Proof of Principle of p24 Detection by Flow Cytometry: Biomaric MIA

Our data indicate that the quantification of heat-denatured p24 by flow cytometry is feasible. The coating efficiency was evaluated after staining the beads with an anti-human IgG-FITC. The analysis of serial dilutions of the IIIB culture supernatant with the Biomaric MIA resulted in reproducible quantification curves (data not shown). The analysis of the p24 standard indicated that the analytical sensitivity of the Biomaric MIA was between 4.6 and 13.7 pg/mL which was lower than the detection limit of both the ultrasensitive Biomaric (1.5–4.6 pg/mL; Fig. 1A), and Perkin Elmer (<0.17 pg/mL; Fig. 2B) EIA. The Biomaric MIA had a wider linear range of HD p24 detection compared with the ultrasensitive Biomaric (3.5 vs. 1.5 logs; Fig. 1A). Background reactivity of the Biomaric MIA and EIA was below the signal of the lowest standard.

Sensitivity of the Biomaric EIA Toward HIV-1 Clades

The Biomaric MIA could detect p24 from HIV culture supernatants derived from HIV-1 subtypes A, B, A/E, A/G, D, F, G, and J with comparable sensitivity between each subtype. A p24 concentration curve for HIV culture supernatants resulted in a broader linear range of the concentration curve using the MIA compared with the ultrasensitive Biomaric or Perkin Elmer EIA (Figs. 1B–1G). Detection of p24 was less sensitive with the Biomaric MIA than with the reference assay for all the strains tested although samples were six-fold more diluted in the EIA as compared with the MIA format.

P24 Detection by Flow Cytometry with Tyramide Amplification Step: Ultrasensitive Biomaric MIA

We next set out to improve the sensitivity of the Biomaric MIA by the addition of a tyramide amplification step. Amplification of p24 detection using the MIA format was not successful using tyramide Oregon green. In contrast, Tyramide-XX–biotin in combination with streptavidin-PE resulted in a signal-amplification three-fold higher than the positive control without any increase in background reactivity (Fig. 2A). The analytical sensitivity improved with tyramide-XX–biotin in combination with Streptavidin-PE (0.51–1.50 pg/mL; Fig. 2B). However, the reference EIA from Perkin Elmer remained the most sensitive (<0.17 pg/mL; Fig. 2B).

DISCUSSION

Cell-free HIV-1 p24 antigen detection using the heat-mediated immune complex disruption procedure has never been applied to flow cytometry before. By using a commercial anti-p24 antibody pair (Biomaric), we succeeded in quantifying HIV p24 with an immunoassay using microspheres as a solid phase. We demonstrated that HIV p24 antigen present in plasma and serum-free samples can be captured, detected, and quantified on beads with flow cytometry. This is in accordance with previous reports describing the application of MIAs for the detection of a variety of targets (reviewed in 10), such as antibodies, pathogens, cytokines, and nucleic acids. MIA are frequently used because of their capacity to detect several analytes at once in a single sample (multiplex), which is much more efficient than running several traditional ELISAs.

The Biomaric MIA resulted in a wider detection range than the ultrasensitive Biomaric EIA. The use of small particles as the solid phase of the assay brings the reactants into closer contact and leads to reaction kinetics approaching liquid phase conditions. Consequently, the amount of captured anti-p24 is larger per bead compared with one micro well of an ELISA plate resulting in more sensitive detection of the antigen. Sensitive detection is particularly important for the quantification of p24 anti-

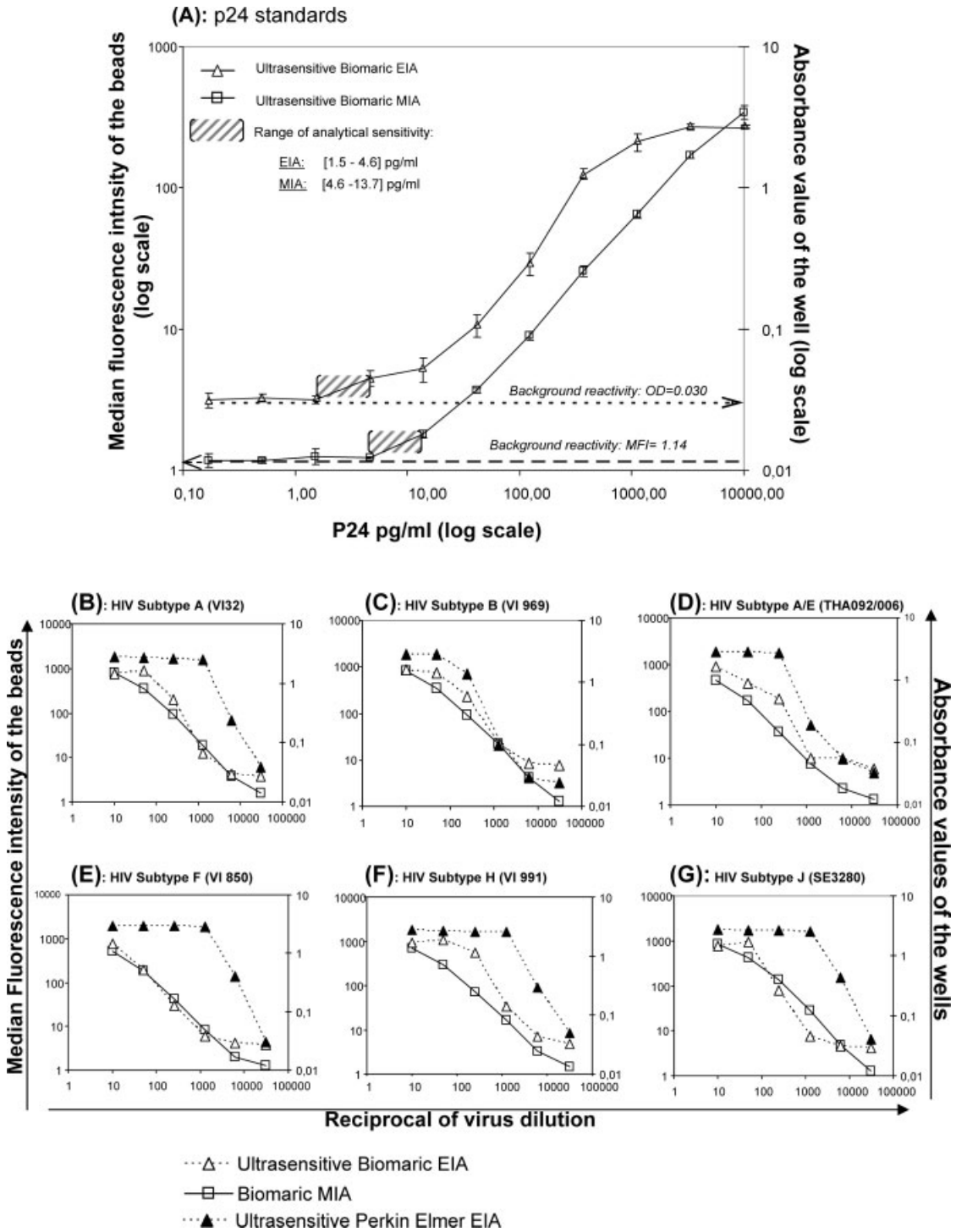

FIG. 1. Detection of p24 with the Biomaric antibody pair using the ultrasensitive EIA and the MIA formats. (**A**) P24 standards: Serial dilutions of the p24 standard from the Perkin Elmer kit were diluted 1 to 6 and heat-treated. P24 quantification was done in parallel and in duplicate using the Biomaric MIA (–□–) and the ultrasensitive Biomaric EIA (–△–). The background reactivity of each assay was below the lowest standard and is indicated by horizontal dotted lines. The analytical sensitivity is defined as the average between the two lowest concentrations of p24 giving a positive signal change and is indicated by the stripped area (▨). (**B–G**) P24 from different HIV-1 subtypes: Heat-denatured HIV culture supernatants from 10 clades of HIV were tested in the Biomaric MIA (–□–), the ultrasensitive Biomaric (- -△- -), and the Perkin Elmer EIA (- -▲- -). Only six representative experiments are displayed. For the ultrasensitive EIA format, HIV supernatants were further diluted 1 to 6 before being tested. Results of the EIA are expressed in absorbance values (left *y* axis) and results from the Biomaric MIA are expressed in median fluorescence intensity (right *y* axis).

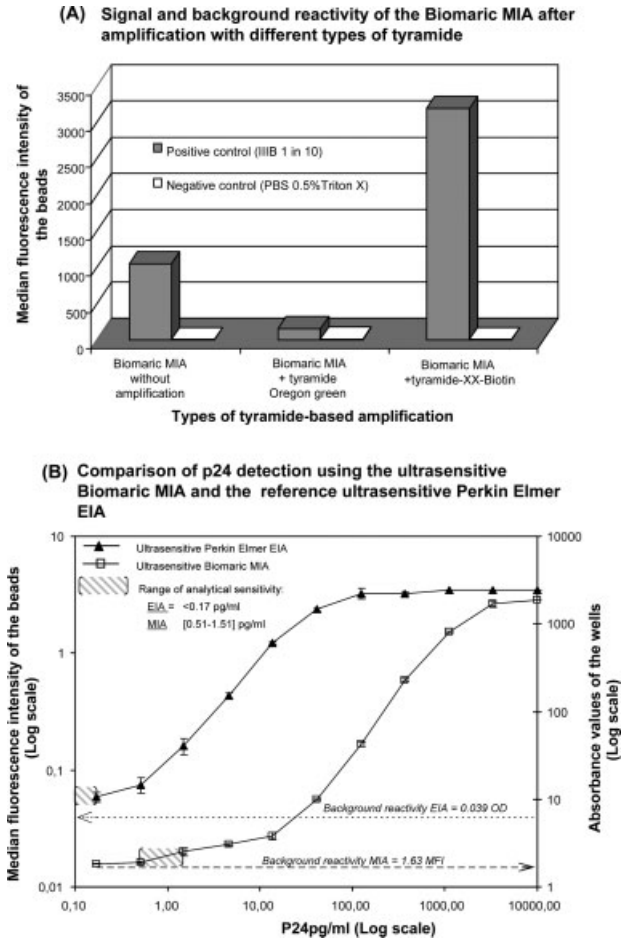

FIG. 2. Development of an ultrasensitive Biomaric MIA. (**A**) Comparison of the signal amplification of the Biomaric MIA using different types of tyramide. IIIB culture supernatant diluted 1 to 10 in PBS + 0.5% triton-X was used as the source of antigen. PBS + 0.5% Triton X was used as the negative control. Samples were not heat-denaturated. Optimum concentration of tyramide Oregon green (diluted 1 in 100) and tyramide-XX–biotin (diluted 1 to 50 and in combination with Streptavidin-PE) were previously determined by titration. Results are expressed in median fluorescence intensity. Tyramide-XX–biotin results in a three-fold amplification of the fluorescence signal of the positive control, whereas tyramide Oregon green seems to inhibit the signal. (**B**) Comparison of p24 detection using the ultrasensitive Biomaric MIA and the reference ultrasensitive Perkin Elmer EIA. Serial dilutions of the p24 standard from the Perkin Elmer kit were diluted 1 to 6 and heat-treated. P24 quantification was done in parallel and in duplicate using the ultrasensitive Biomaric MIA (–□–) and the reference ultrasensitive Perkin Elmer EIA (--▲--). The background reactivity of each assay was below the lowest standard and is indicated by horizontal dotted lines. The analytical sensitivity is defined as the average between the two lowest concentrations of p24 giving a positive signal change and is indicated by the stripped area (▨)

gen in plasma of HIV patients, because HIV load can be very low during ART. One additional advantage of the MIA over the EIA format is higher precision. In the MIA, each bead represents an individual test that runs 10,000 times (number of beads per test), whereas, in ELISA, samples are often tested in duplicate wells. This higher precision is particularly attractive for the quantification of analytes of low-abundance. A number of technical interventions could also further increase the robustness of the assay, such as automation of sample loading and analysis on a microplate as well as the use of software especially designed for the quantification of fluorescence. Another suggestion is to use calibration beads as intratube quality control for the normalization of the fluorescent signal generated on the detection of p24. This could improve the reproducibility and precision of the test in clinical settings.

Amplification of the fluorescence signal using a tyramide-XX–biotin was feasible. Amplification using tyramides directly linked to biotin or to a fluorophor were unsuccessful possibly due to steric hindrance. Amplification with tyramide directly linked to a fluorophor has been described for the detection of cell surface or intracellular proteins of low abundance employing cell phenotyping and flow cytometry (11,12) and experimental settings that require fewer binding steps than our p24 MIA The tyramide amplification step increased the analytical sensitivity of our assay above that of a comparable flow cytometry assay (13). However, the detection limit of the ultrasensitive Biomaric MIA remained inferior to that of the ultrasensitive Perkin Elmer EIA. This inferior detection limit suggests a lower affinity of the Biomaric antibodies for heat-denatured HIV p24 compared with the reference assay: a situation unlikely to be overcome by technical fine tuning. Improvement of the assay for clinical testing should preferably focus on the identification of HD p24 reagents with a comparable or even superior analytical sensitivity than the reference assay from Perkin Elmer. Two such antibody pairs from BioMérieux (including the commercially available Vironostika, data not shown) were identified during a preliminary ELISA-based screening on a large panel of prototype and commercially available anti-p24 antibodies. If these commercially available reagents could be made available for this type of research, the clinical usefulness of the ultrasensitive p24 MIA could be improved significantly.

This report is the first to demonstrate that quantification of HIV p24 antigen using tyramide amplification and the heat-mediated immune complex disruption procedure is feasible using standard flow cytometry. An ultrasensitive MIA based on commercially available anti-p24 antibodies from Biomaric has been developed and can be used for the detection of p24 from a broad range of HIV-1 subtypes.

Although the development of robust clinical assay requires the selection of a more sensitive antibody pair combined with further methodological improvements, these results provide new perspectives in terms of determining viral replication and CD4 count together on a unique flow cytometry platform.

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