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Quantitative Detection of HIV-1 Particles Using HIV-1 Neutralizing Antibody-Conjugated Beads

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The human immunodeficiency virus (HIV) pandemic mainly affects developing countries, where the Joint United Nations Programme on HIV/AIDS (UNAIDS) estimates suggest that less than 1 in 10 people are aware of their HIV sero-status. In order to enhance epidemiological surveys, prevention programs, and therapeutic interventions, development of specific, rapid, and convenient diagnostic detection systems is still warranted. Here we report the direct detection of HIV particles using broadly HIV-1 neutralizing gp120 monoclonal antibody (gp120MAbs)-conjugated magnetic beads (MBs) and fluorescent nanosized polymeric beads (FNBs). The HIV-1 envelope glycoprotein gp120 is anchored to the viral surface through gp41 and mediates entry into target cells by interaction with the main cellular receptor (CD4) and coreceptors (e.g., CCR5 and CXCR4). FNBs conjugated to gp120MAbs (gp120MAbs−FNBs) were used to generate fluorescent signals, whereas MBs conjugated to gp120MAbs (gp120MAbs−MBs) were employed to isolate HIV-1 particles. In presence of HIV-1 particles, addition of gp120MAbs−FNBs and gp120MAbs−MBs leads to the formation of a MBs/HIV-1 particles/FNB complex, which can be easily isolated and concentrated by common magnet separation. We demonstrate the ability of detecting HIV-1 particles specifically and directly using MBs and FNBs with low sample volume (less than 100 μL) and rapidity (less than 1.5 h) without any pretreatment of test samples. The specific binding of FNBs with HIV-1 particles on the surface of MBs was confirmed by fluorescence microscopy and fluorescence-activated cell sorting (FACS). Imaging and FACS analysis revealed the specific and quantitative detection of HIV-1 particles. These results provide proof-of-principle that broadly HIV-1 neutralizing gp120 antibodies coupled to nanobeads can be employed for the direct detection of HIV-1 particles with potential implication for the development of specific, rapid, and convenient diagnostic systems.

Rapid, inexpensive, and easy-to-perform detection assays for pathogenic viruses are important in many applications including clinical diagnostics, vaccine design, treatment, and biological research. In the case of direct virus particle detection, there are only very few reports[1,2] compared to DNA/RNA- or protein-based detection methods.[3−7] Significant improvements have been progressed in the area of molecular detection using polymerase chain reaction (PCR) or enzyme-linked immunosorbent assay (ELISA) techniques. Although these methods offer quite high accuracy and sensitivity in diagnostic settings, they need pretreatment steps of samples to extract target components, prevention of contamination, and expensive tools to perform the assays.[8] Therefore, more cost-effective and rapid detection assays in terms of virus particles rather than components of the virus are in demand, especially in resource-poor setting areas such as undeveloped countries, in which virus-related diseases are mainly prevalent.

The World Health Organization (WHO)/Joint United Nations Programme on HIV/AIDS (UNAIDS) estimates that 40 million people are living with human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS) infection to date.[9] Current estimates suggest that less than 1 in 10 people are aware of their HIV sero-status.[10] Therefore, highly specific, rapid, and convenient HIV-1 detection systems are required to facilitate identification of infected individuals, preventing them from further infecting others, monitoring disease progression of infected individuals, and investigating clinical trials of new drugs or vaccine candidates. Toward this goal, many HIV-1 detection assays were proposed and used in clinical and laboratory areas. At present the most widely used and U.S. Food and Drug Administration (FDA)-approved HIV-1 screening test kit, OraQuick Advance, is based on IgG antibody tests in oral fluid samples based on a

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salivary component and the oral mucosal transudation.[10] Although these oral fluid tests offer accurate, feasible, and clinically acceptable performance, the test results are considered preliminary because of hypermutable features of HIV-1 and low-level antibody presence in the antibody-free window period.[8,10,11] Another method is based on detection of the HIV-1 core antigen.[12,13] Further, nucleic acid based tests (PCR or real-time PCR (RT-PCR)), which target highly conserved regions of the HIV-1 *gag* gene, are widely used to confirm an HIV infection.[14,15] These methods are widely accepted and used for the diagnosis of HIV infection but rely rather on the detection of byproducts of an HIV-1 infection or their components than detection of the virus particle itself. The byproduct detection methods are usually expensive, time-consuming, labor-intensive, and complicated in their protocol. To our knowledge, there is currently no report about the direct detection of HIV-1 particles, which may offer a convenient and reliable detection method without complicated sample preparation.

HIV infects target cells that express CD4 on their surface. HIV infection requires a fusion of viral and cellular membranes. This process is accomplished by the viral envelope glycoprotein (gp120, gp41) and receptors (CD4 and coreceptors, such as CXCR4 or CCR5) on the target cell.[16−18] For an HIV-1 vaccine, the envelope glycoproteins (Envs) gp120 and gp41 are the only virus-encoded determinants that are present on the virus surface. Conserved regions in these proteins are candidate targets for the development of antibodies that can neutralize a wide array of circulating HIV-1 isolates.[9,18] So far, only four broad HIV-1 neutralizing antibodies have been well defined—two against gp120 (b12 and 2G12) and two against gp41 (2F5 and 4E10)—and each of these was elicited by natural infection. Considering the HIV-1 infection process mediated by gp120 and CD4 binding and endeavor of vaccine development, we hypothesized that gp120MAbs also might be useful as probes for efficient and direct diagnosis of HIV-1 particles. From this point of view, broadly HIV-1 neutralizing antibody should be feasible for identification of a broad range of HIV-1 isolates.[9,16,18−20]

Herein, we report the development of an HIV-1 particle diagnostic system using broadly HIV-1 neutralizing gp120MAbs (2G12) conjugated to magnetic beads (MBs) and fluorescent nanosized polymeric beads (FNBs) that allows direct capture and quantification of HIV-1 particles without any pretreatment of the samples. Specific and nonspecific binding of HIV-1 particles with MBs and FNBs was systematically characterized at each chemical linker or antibody conjugation step. HIV-1 detection mediated by MBs and FNBs was analyzed using fluorescence-activated cell sorting (FACS) and fluorescence microscopy coupled quantitative image analysis. To our knowledge, this is the first report about the direct detection of HIV-1 particles using broadly HIV-1 neutralizing antibody-conjugated beads.

EXPERIMENTAL SECTION

Materials. Carboxylated FNBs (mean diameter, ~92 nm, Dragon green dye (ex 480 nm, em 520 nm) encapsulated polystyrene beads) and carboxylated MBs (mean diameter, ~8.31 μm, COMPEL magnetic) were purchased from Bangs Laboratories, Inc. (Indiana, U.S.A.). The broadly HIV-1 neutralizing gp120 monoclonal antibody (gp120MAb), 2G12, was purchased from Polymun Scientific (Vienna, Austria). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide (sulfo-NHS), and 2-(morpholino) ethanesulfonic acid (MES) were purchased from Pierce (Illinois, U.S.A.). Human serum samples (HS2−HS4) were kindly provided by Dr. Sungae Cho (Institut Pasteur Korea) or purchased from KOMA Biotech, Korea (HS1). Fetal bovine serum (FBS) was purchased from Invitrogen (California, U.S.A.). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Production of HIV-1 Particles. HEK293 cells (America Type Culture Collection, CRL-1573) were cotransfected by $CaCl_2$ transfection with two different plasmids, pCMV-dR8.74 and pDOLHIVenv,[21,22] which contain Gag and Pol, and Env components of HIV-1, respectively. Cotransfected HEK293 cells were cultured in DMEM medium supplemented with 10% FCS at 37 °C in 5% CO_2. After 2 days, supernatant was collected and HIV-1 particles were concentrated by ultracentrifugation (26 000 rpm for 2 h at room temperature). The concentrated particles were resuspended in DMEM and kept at −80 °C until use. The concentration of produced virus-like particles (VLPs) was determined by p24 ELISA assay kit (Perkin-Elmer, Boston, MA).

Conjugation of gp120 Monoclonal Antibody with MBs and FNBs. Carboxylated MBs (7.82×10^5 particles) were first washed with 100 mM MES buffer (pH 5.9) and then mixed with sulfo-NHS (46 mM) and EDC (26 mM) solution. MBs were incubated for 30 min at room temperature under vigorous shaking. After incubation, MBs were separated with a common magnet and then washed three times with 100 mM MES buffer (pH 5.9). Washed MBs were mixed with gp120MAbs (2G12, 83 pmol) and incubated for 3 h under vigorous shaking at room temperature. After incubation with 2G12, MBs were washed three times with 10 mM sodium phosphate buffer (PBS, pH 7.2). The amount of immobilized gp120MAbs was then calculated by subtracting the amount of gp120MAbs determined in the residual solutions and washings from the total amount of gp120MAbs used in the immobilization procedure using the BCA (bicinchoninic acid) assay kit (Pierce, Illinois). For the capping of unreacted EDC, 100 mM glycine (pH 8.0) was mixed

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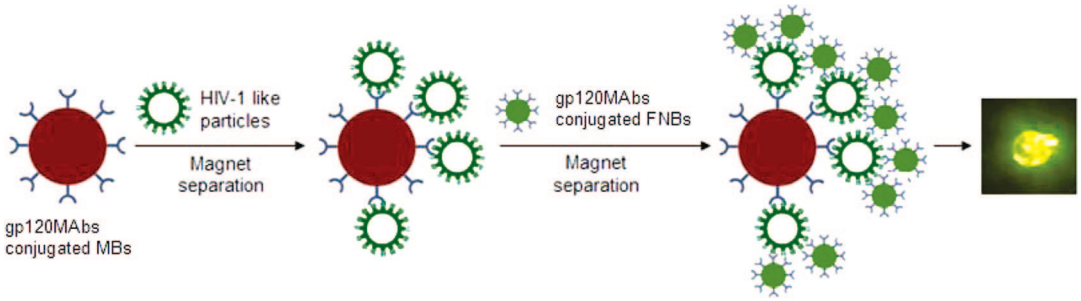

Figure 1. Scheme of HIV-1 detection method using broadly HIV-1 neutralizing gp120 antibody-conjugated beads. The particle detection comprised three steps: (i) HIV-1 particles were captured and isolated by gp120MAbs−MBs, (ii) gp120MAbs−FNBs were added and allowed to bind additional gp120 molecules on HIV-1 particles, and (iii) MBs/HIV-1 particles/FNBs complexes were further purified and subjected to fluorescent signal analysis.

with gp120MAbs-conjugated MBs (gp120MAbs−MBs) and incubated for 30 min at room temperature. After three more washing steps with 10 mM PBS buffer, gp120MAbs−MBs were resuspended with 10 mM PBS buffer and stored at 4 °C until use. For carboxylated FNBs conjugation with gp120 MAbs, carboxylated FNBs (2.31×10^{11} particles) were suspended in 100 mM MES buffer (pH 5.9) and mixed with sulfo-NHS (46 mM) and EDC (26 mM) solution. After 30 min of incubation and brief sonication, EDC−NHS-conjugated FNBs were centrifuged (13 000 rpm) for 30 min at room temperature and washed with MES buffer. An amount of 166 pmol of gp120MAbs was mixed with EDC−NHS-conjugated FNBs, and the mixture was incubated for 3 h under vigorous shaking at room temperature. After 3 h of incubation gp120MAbs-conjugated FNBs (gp120MAbs−FNBs) were separated by centrifugation (13 000 rpm) for 30 min at room temperature. The centrifugation step was repeated two more times to remove unconjugated gp120MAbs as resuspending debris with 100 mM MES buffer (pH 5.9). For the capping of unreacted EDC, 100 mM glycine (pH 8.0) was mixed with gp120MAbs−FNBs and incubated for 30 min. After three more washing steps with 10 mM PBS buffer, gp120MAbs−FNBs were resuspended in 10 mM PBS buffer and stored at 4 °C until use.

HIV-1 Detection Assay. Five microliters of gp120MAbs−MBs ($\sim 2 \times 10^4$ particles) was mixed with 10 μL of VLPs stock solution diluted in human serum samples or FBS and 85 μL of 10 mM PBS−TBN buffer (pH 7.2, 100 mL based 10 mM PBS buffer with 0.1 g of BSA, 0.02 g of sodium azide, and 0.02 mL of Tween 20). The mixture was allowed to react under vigorous shaking at room temperature for 30 min. The MBs were separated using common magnet and washed three times with 10 mM PBS buffer (pH 7.2). The MBs with captured VLPs on the surfaces were resuspended in 90 μL of 10 mM PBS−TBN buffer (pH 7.2) and were allowed to react with 10 μL ($\sim 1.15 \times 10^{10}$ particles) of gp120MAbs−FNBs with vigorous shaking for additional 30 min at room temperature. Finally, the MBs/VLPs/FNBs complex was separated by magnet, washed three more times, and concentrated to 10 μL with DI water. Concentrated samples were used for fluorescence microscopy (Olympus, Japan) and FACS (BDFACSCanto II, BDscience, U.S.A.) analyses. Arbitrary pixel intensity of magnetic beads in microscopic images was analyzed using ImageJ (http://rsb.info.nih.gov/ij/) software. The correlation of the quantity of VLPs in the samples with recovered fluorescence intensities of FNBs bound to MBs by FACS and image-based analyses, respectively, was analyzed by nonlinear regression in terms of a simple single-site biding model for the antibody−antigen interaction[20] using SigmaPlot (Systat Software, Inc., Illinois).

RESULTS AND DISCUSSION

The HIV-1 particle detection method described herein relies on the immunogenic ability between gp120 on the HIV-1 envelope and the broadly HIV-1 neutralizing gp120 antibody, 2G12. As illustrated in Figure 1, the HIV-1 detection comprises three steps: (i) HIV-1 particles were captured and isolated by gp120MAbs−MBs, (ii) gp120MAbs−FNBs were added and allowed to bind additional gp120 molecules on HIV-1 particles, and (iii) MBs/HIV-1 particles/FNBs complexes were further purified and subjected to fluorescent signal analysis.

To explore the feasibility of neutralizing antibody-conjugated beads for the direct and quantitative detection of HIV-1 particles, we employed HIV-1 VLPs produced by cotransfection of pCMV-dR8.74 (expressing *gag* and *pol* genes) and pDOLHIVenv (expressing the *env* gene) into HEK293 cells. The *gag* gene encodes the structural proteins of the viral capsid which are sufficient for particle assembly. The Pol protein contains viral enzymes that are required for infectivity (e.g., the viral protease required for maturation of released virions). The *env* gene encodes the proteins required for viral binding to and entry into the host cell. This polyprotein is cleaved in the Golgi compartment by a cellular protease to produce two mature glycoproteins, the surface gp120 and transmembrane protein, gp41. These two proteins are associate together to form the envelope protein complex on the surface of the plasma membrane that is incorporated into the viral envelope during budding.[23] Thus, the VLPs represent genuine virions that are, however, noninfectious because of the absence of viral genomic components inside the particles.

For the conjugation of gp120MAbs with MBs and FNBs, an EDC−NHS reaction was applied, which is well established for the coupling of amine and carboxyl groups. EDC was reacted with a carboxyl group on MBs or FNBs to form an amine-reactive *O*-acylisourea intermediate. The addition of sulfo-NHS stabilizes the intermediate by converting it to an amine-reactive sulfo-NHS ester. The amine-reactive ester permits a two-step cross-linking process that allows linking of the carboxyl groups with the amino groups of the antibody.[24] Successful conjugation of the antibody with MBs and FNBs was controlled by gel electrophoresis and

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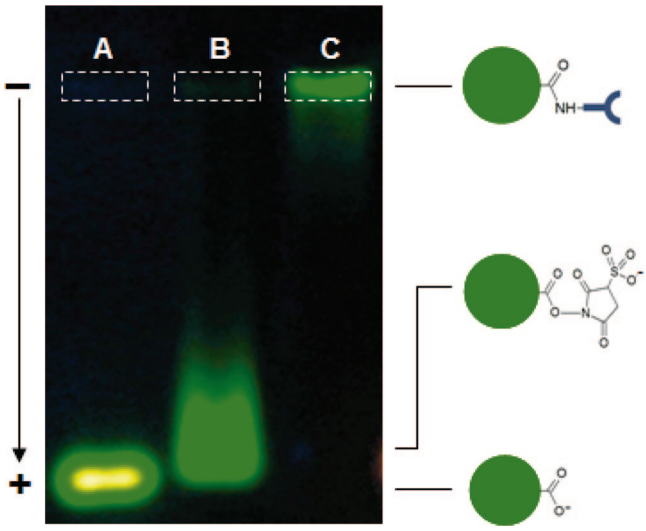

Figure 2. Gel electrophoresis (0.5% agarose in 0.5× TBE) of FNBs: (A) unreacted carboxyl FNBs, (B) EDC−NHS coupled FNBs, and (C) gp120MAbs−FNBs. White dotted boxes denote sample loading wells.

BCA protein assay, respectively. As shown in Figure 2, the unreacted carboxyl FNBs and EDC−NHS-conjugated FNBs migrated far from the loading well as expected due to the negative charges on the surface of FNBs by protonation of carboxylic groups or amine-reactive ester groups. Importantly, gp120MAbs−FNBs were retained at the well position and did not migrate into the gel, confirming that gp120MAbs−FNBs had lost their negative charges after cross-linking. Though MBs also have negative charges of carboxyl groups on their surface, antibody conjugation cannot be monitored by gel electrophoresis due to their size and weight. Thus, conjugation of gp120MAbs was analyzed by colorimetric detection using the BCA protein assay kit. The quantity of conjugated gp120MAbs was determined by the difference of UV absorbance values of the BCA solution before and after conjugation of gp120MAbs with MBs. This revealed that ~0.5 μg of gp120MAbs associated with 7.82×10^5 MB particles corresponding to an average number of $\sim 3.2 \times 10^6$ gp120MAbs per MB (data not shown).

In the next step, the specificity of gp120MAbs−MBs and gp120MAbs−FNBs to detect HIV-1 VLPs was investigated. As control, carboxylated FNBs and carboxylated MBs were mixed with or without VLPs, and then MBs were separated and analyzed by fluorescence microscopy. In both cases no fluorescent signals could be detected, ruling out any unspecific binding of the HIV-1 VLPs to the beads (Figure 3, parts A and B). Similarly, when EDC−NHS coupled FNBs and EDC−NHS coupled MBs were mixed with or without VLPs or when gp120MAbs−FNBs and gp120MAbs−MBs were incubated without VLPs, no fluorescent signals could be observed (Figure 3C−E). In sharp contrast, when HIV-1 VLPs were incubated with gp120MAbs−FNBs and gp120MAbs−MBs fluorescent signals were easily detectable, whereas incubation of gp120MAbs−FNBs and gp120MAbs−MBs with bacteriophage-like particles (total $\sim 2 \times 10^4$ particles, Asuragen, Texas) were negative, demonstrating the specificity of the interaction (Figure 3, parts F and G). Thus, 2G12-conjugated beads are a suitable and specific tool for the detection of HIV-1 particles.

To validate the ability of this method for quantitation of viral titers, serial dilutions of HIV-1 VLPs were prepared in human

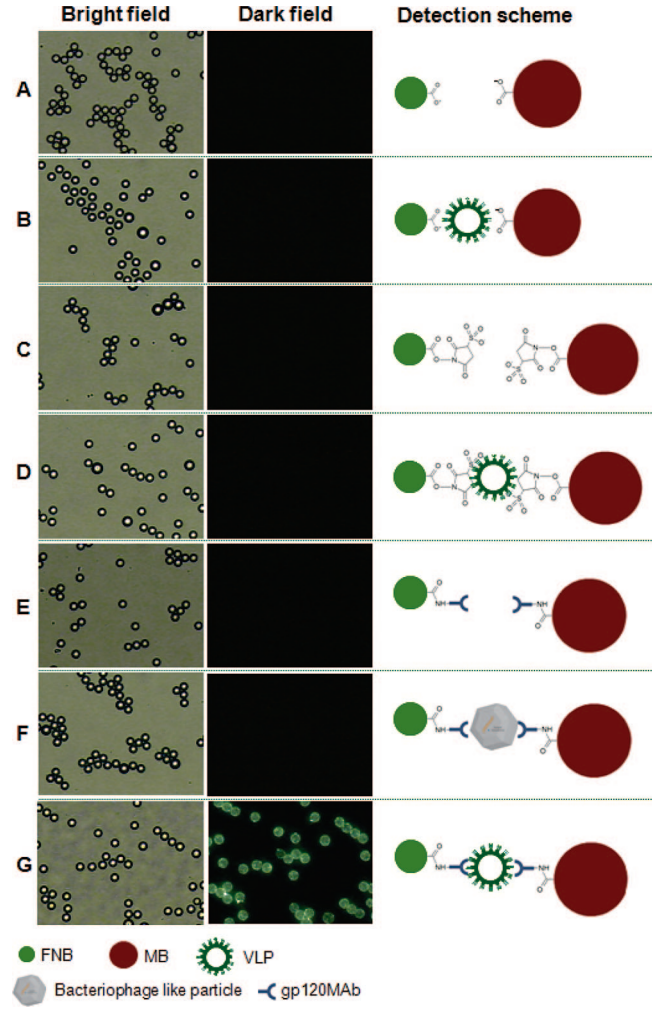

Figure 3. Specificity of VLP detection; left boxes denote bright-field images, and right boxes are dark-field images from fluorescence microscopy: (A) carboxyl FNBs/carboxyl MBs, (B) carboxyl FNBs/VLPs/carboxyl MBs, (C) EDC−NHS coupled FNBs/EDC−NHS coupled MBs, (D) EDC−NHS coupled FNBs/VLPs/EDC−NHS coupled MBs, (E) gp120MAbs−FNBs/gp120MAbs−MBs, (F) gp120MAbs−FNBs/bacteriophage-like particles/gp120MAbs−MBs, and (G) gp120MAbs−FNBs/VLPs/gp120MAbs−MBs.

serum (HS1) and the mean fluorescence intensity (MFI) of isolated MBs/VLPs/FNBs complexes was determined by FACS analysis. As can be seen in Figure 4A−F, all VLP-containing samples demonstrated significant fluorescent profiles compared to the negative control samples, and the assay proved to be robust with low variability (Figure 4G). Further, a nonlinear regression analysis assuming a single-site binding mode for the antibody−antigen interaction strongly supports the notion that the intensity of FNBs bound to MBs correlates well with the quantity of VLPs in the samples (Figure 4G). Comparable results were obtained when arbitrary pixel intensities of the isolated MBs/VLPs/FNBs complexes were determined by microscopic examination followed by quantitative image analysis using ImageJ software (Figure 4H). Since sera are complex and variable fluids, we then prepared serial dilutions of HIV-1 VLPs in different serum samples (four human sera and FBS) and determined the MFI of isolated MBs/VLPs/FNBs complexes by FACS analysis. Albeit there was a notable difference of relative fluorescence signals, we importantly observed a dose-

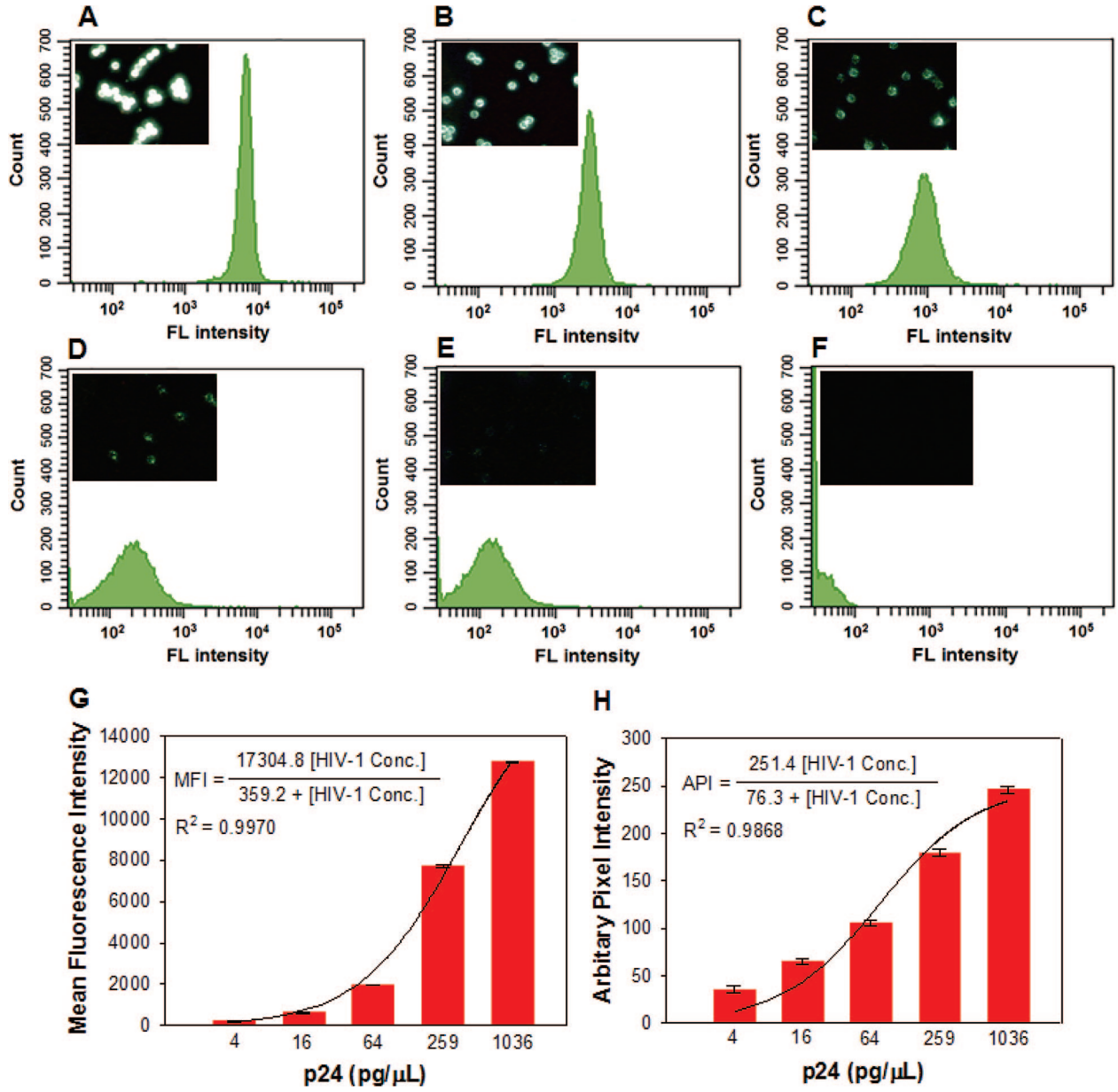

Figure 4. Quantitative analysis of gp120MAbs−FNBs/VLPs/gp120MAbs−MBs using FACS and fluorescence microscopy. Serial dilutions of HIV-1 VLPs corresponding to (A) 1036, (B) 259, (C) 64, (D) 16, (E) 4, and (F) 0 pg/μL of p24 capsid antigen were prepared in human serum (KOMA Biotech, Korea), and the mean fluorescent intensity (MFI) of isolated MBs/VLPs/FNBs complexes was determined by FACS analysis. Representative FACS profiles for each concentration from at least three independent experiments are shown. Insets are representative fluorescence microscopy images of the corresponding samples. (G) Bar graph illustration of the MFI depending on the HIV-1 p24 core antigen concentration of three independent experiments (±STDEV). The superimposed black line represents the curve fit from a nonlinear regression analysis assuming a single-site binding equation for the antibody−antigen interaction as indicated. (H) Bar graph illustration of the arbitrary pixel intensity (API) depending on the HIV-1 p24 core antigen concentration of 5 representative beads/picture (±STDEV). The superimposed black line represents the curve fit from a nonlinear regression analysis assuming a single-site binding equation for the antibody−antigen interaction as indicated.

dependent increase in the MFIs in all cases tested, which correlated well with the p24 concentration of the samples (Figure 5). Together these results demonstrate that this approach is feasible to isolate HIV-1 VLPs from human serum by using first magnetic beads and by performing a subsequent detection of the VLPs with fluorescent beads which can be quantitatively analyzed by both, fluorescence microscopy and flow cytometry.

CONCLUSION

In summary, the results provide proof-of-concept that broadly HIV-1 neutralizing antibody-conjugated beads can be employed for the specific and quantitative detection of HIV-1 particles. The procedure is fast (within 1.5 h) and simple with high specificity and without the need of pretreatment of samples. As compared to DNA/RNA- or protein-based analysis the direct detection of HIV-1 particles offers several advantages. By eliminating sample treatment steps for extracting DNA/RNA or protein, the HIV-1

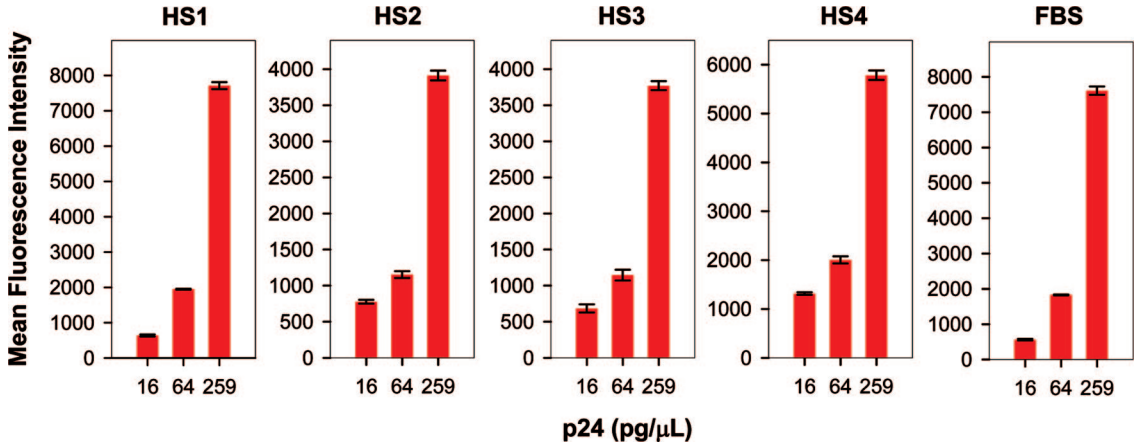

Figure 5. Specific detection of VLPs in different sera. Dilutions of HIV-1 VLPs corresponding to p24 capsid antigen concentrations as indicated were prepared in four different human sera (HS1−HS4) and in FBS. Bar graph illustration of the mean fluorescence intensity (MFI) depending on the HIV-1 p24 core antigen concentration of three independent experiments (±STDEV) is shown.

particle detection is easy to perform with minimal need of laboratory equipment or infrastructure. Further, the assay is based just on mixing and separation, and therefore minimal expertise is required to perform the detection assay. We also demonstrate that the isolated VLPs can be quantified not only by FACS analysis but also by microscopic examination followed by quantitative image analysis. Given the potential application as a diagnostic tool kit, we believe that an image-based detection system could have some advantage compared to FACS analysis—in particular in resource-poor settings. Magnetic separation is effective and specific for the isolation and detection of biological material in complex samples and the use of broadly neutralizing antibodies may be applicable to detect a wide range of HIV-1 clades.[25] It is also noteworthy that magnetic beads are useful tools to enrich biological materials from rather large volumes, which may facilitate the detection of minor variants usually difficult to detect due to sampling bottlenecks. For instance, the magnetic bead separation of HIV-1 particles described here followed by PCR analyses could also be employed in genotyping approaches to determine the appearance or prevalence of drug resistant HIV-1 variants, and it may be possible to couple a cocktail of different neutralizing antibodies to the beads, to be able to isolate escape mutants for particular antibodies. We wish also to emphasize that the use of antibody-conjugated beads for the detection of surface proteins may be applicable to other pathogens either alone or as a multiplexing system with appropriate combinatorial design of detection probes and the results presented here may be a first step for the development of a rapid, inexpensive, and easy-to-perform diagnostic tool kit for HIV-1 particle detection.

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