Inexpensive Designer Antigen for Anti-HIV Antibody Detection with High Sensitivity and Specificity

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A novel recombinant multiepitope protein (MEP) has been designed that consists of four linear, immunodominant, and phylogenetically conserved epitopes, taken from human immunodeficiency virus (HIV)-encoded antigens that are used in many third-generation immunoassay kits. This HIV-MEP has been evaluated for its diagnostic potential in the detection of anti-HIV antibodies in human sera. A synthetic MEP gene encoding these epitopes, joined by flexible peptide linkers in a single open reading frame, was designed and overexpressed in Escherichia coli. The recombinant HIV-MEP was purified using a single affinity step, yielding >20 mg pure protein/liter culture, and used as the coating antigen in an in-house immunoassay. Bound anti-HIV antibodies were detected by highly sensitive time-resolved fluorometry, using europium(III) chelate-labeled anti-human antibody. The sensitivity and specificity of the HIV-MEP were evaluated using Boston Biomedica worldwide HIV performance, HIV seroconversion, and viral coinfection panels and were found to be compatible with those of commercially available anti-HIV enzyme immunoassay (EIA) kits. The careful choice of epitopes, high epitope density, and an E. coli-based expression system, coupled with a simple purification protocol and the use of europium(III) chelate-labeled tracer, provide the capability for the development of an inexpensive diagnostic test with high degrees of sensitivity and specificity.

Human immunodeficiency virus (HIV) is a lentivirus of the family Retroviridae, whose members characteristically have an RNA genome within a capsid and a lipid envelope. HIV infection induces a profound immune dysfunction, with abnormalities in every arm of the immune system, resulting in AIDS (5). In 2007, there were 2.7 million new HIV infections and 2 million HIV-related deaths. Globally, there were an estimated 33 million people living with HIV in 2007. India is one of the largest and most populated countries in the world, with a population of over 1 billion. Of this number, it is estimated that around 2.4 million Indians were living with HIV in 2007 (26). The genes of HIV are located in the central region of the proviral DNA and encode at least nine proteins. These proteins are divided into three classes: the major structural proteins (Gag, Pol, and Env), the regulatory proteins (Tat and Rev), and the accessory proteins (Vpu, Vpr, Vif, and Nef) (11). The gag gene of HIV type 1 (HIV-1) encodes a polyprotein precursor, p55, which is cleaved by the virus-encoded protease into three proteins, p24, p17, and p15. Linear B-cell epitopes have already been identified within p24 (14). The antigen p24 is of special significance because of its ability to be expressed first in body fluids after HIV-1 infection. The linear immunodominant epitope of p24 serves as an important diagnostic intermediate to detect antibodies to HIV-1 in human sera (23). The envelope glycoproteins (gp), gp41 of HIV-1 and gp36 of the closely related HIV-2, are highly immunogenic and are important diagnostic intermediates for the detection of antibodies to these viruses in human sera (17, 24). HIV-1 comprises three lineages, denoted M, N, and O (22). HIV-2 and divergent forms have been detected in West African or West Africa-related patients with AIDS (7–9). Several enzyme immunoassay (EIA)-based diagnostic kits are available on the market for the detection of antibodies to HIV in human sera. These anti-HIV EIA kits use synthetic peptides and/or recombinant proteins mainly from the envelope gp of HIV-1 group M, HIV-1 group O, and HIV-2. The fourth-generation kits also have antibodies to p24 antigen. The requirement of multiple peptides and/or multiple recombinant proteins for reliable diagnosis of HIV infections adds to the cost of these EIA kits. The high cost of anti-HIV EIA kits becomes prohibitive for routine use in many developing countries, precluding early detection and prevention of new infections (18, 25, 27). We have designed a single recombinant multiepitope protein (MEP) antigen, consisting of several immunodominant, linear, and conserved virus-specific epitopes from structural proteins of HIV-1 and HIV-2. DNAs encoding these epitopes have been assembled in tandem in a single open reading frame, with intervening sequences encoding flexible linkers, and expressed in Escherichia coli. A polyhistidine tag has also been included which allows for facile purification of recombinant MEP by Ni-NTA chromatography. The purified protein has been used as the coating antigen for developing an anti-HIV indirect immunoassay. We have evaluated the performance of this assay with that of other multiple-antigen-based immunoassay kits currently available on the market, using well-characterized commercially available serum panels.
MATERIALS AND METHODS

Materials. E. coli host strains DH5α and BL21(DE3) were purchased from Invitrogen Life Technologies, Carlsbad, CA. Plasmid vector pET-32a(+) was obtained from Novagen, Madison, WI. The synthetic codon, optimized for E. coli expression, encoding the recombinant HIV-MEP (r-HIV-MEP) was custom synthesized by Geneart, Regensburg, Germany. Restriction endonucleases, calf intestine alkaline phosphatase, and T4 DNA ligase used in all routine cloning and transformation experiments were procured from MBI Fermentas, Burlington, Canada. Tag polymerase for PCR screening was an in-house preparation. Ni-NTA superflow resin was purchased from Qiagen, Maryland. Goat anti-human IgG was purchased from Pierce, Rockford, IL. Isopropyl β-D-thiogalactoside (IPTG) was procured from Calbiochem-EMD Biosciences, La Jolla, CA. Well-characterized international serum panels were purchased from Boston Biomedica Inc. (BBI), now SeraCare Life Sciences Inc., Milford, MA. The BBI panels were the worldwide HIV performance panel (WWRB 302-01 to WWRB 302-30), HIV seroconversion panel (PRB 931-01 to PRB 931-09), and viral coinfection panel (PCA 201-01 to PCA 201-25). The europium(III) chelate, Eu3+/9d-chelate, was synthesized as a BamHI/HindIII fragment in the Geneart vector pPCRscript. Restriction endonucleases, Taq polymerase for PCR screening was an in-house preparation, and full description of fluorescent properties of the Eu3+/9d-chelate have been published previously (15, 19). Goat anti-human IgG was labeled using a 40-fold molar excess of the Eu3+/9d-chelate. The labeling and removal of excess free label were performed essentially as described before (19). The protein concentration of the labeled antibody preparation was determined by Bradford assay (3). The level of label incorporation was determined to be 2.6 Eu3+/9d-chelate per antibody. Bovine serum albumin (BSA) and sodium azide were added to final concentrations of 0.1% and 0.05%, respectively. The solution was filtered through a 0.22-μm membrane and stored at 4°C until used further.

In-house indirect HIV immunoassay. Time-resolved fluorometry (TRF) measurements of Eu3+/9d-chelate-labeled anti-HIV antibody in indirect immunoassays with r-HIV-MEP as the capture antigen were made using a Victor®N 1420 Multilabel counter (Perkin Elimer, Singapore), which allows the measurement directly from a solid phase. Briefly, 5 μg/ml of r-HIV-MEP was prepared in coating buffer (0.1 M carbonate-bicarbonate buffer, pH 9.6) and 100 μl of this was added into each well of a 96-well plate and incubated overnight at 37°C. The wells were aspirated and blocked with 300 μl of blocking buffer (37.5 mM Tris-HCl, pH 7.75, 25% goat serum, 115 mM NaCl, 0.05% NaN3, 0.038% Tween 40, 15 μM EDTA, 1.38% BSA) and incubated for 2 h at room temperature with shaking. The wells were washed two times using COLUMBUS Plus-BASIC (TECAN, Göttingen, Austria) with wash buffer (10 mM KH2PO4, 40 mM K2HPO4, pH 7.2, 150 mM NaCl, 0.1% Tween 20, 0.5 M KCl). After washing, 2 μl of each serum sample in 50 μl assay buffer (37.5 mM Tris-HCl, pH 7.75, 25% goat serum, 115 mM NaCl, 0.5 M KCl, 0.05% NaN3, 0.038% Tween 40, 0.1% Triton X-100, 15 μM EDTA, 0.38% BSA) was incubated in each well for 30 min at room temperature with shaking. The wells were washed four times with wash buffer. One microgram per milliliter of Eu3+/9d-chelate labeled anti-human antibody was made in assay buffer, and 50 μl of this was added into each well and incubated for 30 min at room temperature with shaking. The wells were washed seven times with wash buffer, and TRF measurement was performed. The solution was filtered through a 0.22-μm membrane and stored at 4°C until used further.

RESULTS

Design of r-HIV-MEP antigen. To design a MEP that could be of diagnostic utility, linear and conserved immunodominant epitopes, known to elicit anti-HIV antibodies, were selected based on published literature, summarized in Table 1 (10, 12, 14). These epitopes were from HIV-1 p24 and the Env antigens of HIV-1 and HIV-2. The r-HIV-MEP was designed by linking these epitopes in tandem using (Gly-Gly-Gly-Gly) linkers (20). The r-HIV-MEP gene was inserted into the expression vector pET-32a(+) in frame with the vector-encoded thioredoxin gene and six-His tag-encoding sequence, under the control of the tightly regulated T7 promoter. This expression vector was transformed into E. coli strain BL21(DE3). Expression and purification of r-HIV-MEP. Transforms harboring the r-HIV-MEP plasmid were expression screened to choose a clone that expressed r-HIV-MEP maximally. As the r-HIV-MEP antigen, codon optimized for expression in E. coli (21), was custom synthesized as a BamHI/HindIII fragment in the Geneart vector pPCRscript. Regions of very high (>60%) or very low (<30%) GC content, internal TATA boxes, chi-site stretches, internal ribosomal entry sites, AT-rich or GC-rich sequence stretches, repeat sequences, and RNA secondary structures were avoided where possible. The lengths of individual epitopes varied from 28 to 51 amino acid (aa) residues, and the adjacent epitopes were joined together by flexible tetraethylene glycol (Gly-Gly-Gly-Gly) linkers (20). The r-HIV-MEP gene was inserted into the expression vector pET-32a(+), in frame with the vector-encoded thioredoxin gene and six-His tag-encoding sequence, under the control of the tightly regulated T7 promoter. This expression vector was transformed into E. coli strain BL21(DE3).

<table>
<thead>
<tr>
<th>Virus Type</th>
<th>Epitope Position in HIV Proteins</th>
<th>% Positivity with Patient Sera</th>
<th>Reference</th>
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<td>HIV-1 p24</td>
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<td>14</td>
</tr>
<tr>
<td>HIV-1 group O gp41</td>
<td>aa 580–616 of gp160 (863)</td>
<td>84</td>
<td>10</td>
</tr>
<tr>
<td>HIV-2 gp36</td>
<td>aa 587–614 of gp160 (858)</td>
<td>100%</td>
<td>12</td>
</tr>
<tr>
<td>HIV-1 group M gp41</td>
<td>aa 580–625 of gp160 (853)</td>
<td>100</td>
<td>10</td>
</tr>
</tbody>
</table>

* Numbers in parentheses indicate the total number of amino acid residues of the corresponding full-length proteins.

** ND, not done.

† In combination with HIV-1 gp41 (31 aa) and p24 (146 aa).

TABLE 1. List of HIV-specific immunodominant epitopes selected from the literature in designing the r-HIV-MEP antigen.
IPTG induction from a 1-liter shake-flask culture. As localization experiments showed that the r-HIV-MEP was associated exclusively with the insoluble pellet fraction of lysates, we solubilized it using guanidinium and purified it under denaturing conditions by Ni-NTA affinity chromatography (data not shown). We obtained ~21 mg of >95% purified protein from a liter of induced culture.

**Evaluation of the r-HIV-MEP with Boston Biomedica serum panels.** Next, we sought to establish an in-house anti-HIV

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**FIG. 1.** The r-HIV-MEP antigen designed for this study. (A) Computer-generated graphic visualization (http://www.sbg.bio.ic.ac.uk/~3dpssm) of r-HIV-MEP. (B) Complete nucleotide (lowercase letters) and predicted amino acid (capital letters) sequences of the r-HIV-MEP gene showing four epitopes (aa 1 to 51, p24 of HIV-1; aa 56 to 92, gp41 of HIV-1 group O; aa 97 to 124, gp36 of HIV-2; and aa 129 to 174, gp41 of HIV-1 group M) linked together with flexible tetraglycyl linkers (underlined). The asterisk indicates the engineered stop codon.

**FIG. 2.** Multiple sequence alignment of the four r-HIV-MEP epitopes with the corresponding epitopes of different HIV types, groups, and subtypes (http://bioinfo.genotoul.fr/multalin/multalin.html). The black dots indicate identical residues. Variants are indicated by the standard single-letter amino acid code. Letters in the virus names indicate subtypes. In the case of HIV-1 group O gp41, the alignment has been done with different isolates within the group.
indirect immunoassay using the purified r-HIV-MEP. In this assay, the purified r-HIV-MEP antigen was used to capture anti-HIV antibodies in sera. Bound anti-HIV antibodies were detected by TRF using anti-human antibody labeled with Eu³⁺-9d-chelate. To establish the specificity of r-HIV-MEP protein as an intermediate for anti-HIV immunoassay, more than 50 HIV-negative human serum samples were evaluated. The results demonstrated that r-HIV-MEP did not exhibit any false positivity with normal human serum samples. These results unequivocally established the high degree of specificity of r-HIV-MEP protein for the detection of anti-HIV antibodies.

Next, 57 serum samples from various well-characterized BBI panels were used to evaluate our in-house r-HIV-MEP-based anti-HIV immunoassay. Table 2 compares the ability of our in-house assay to detect early seroconversion with those of other commercial kits using a set of nine sera constituting the HIV seroconversion panel (PRB 931). The earliest time point at which seroconversion is detected in this panel is at 28 days, using the in-house assay, represented by panel member 6. In addition to our in-house assay, only one kit, namely, the Abbott HIV-1/2 kit, out of the five commercial kits tested could pick up this member. We assessed the sensitivity of the r-HIV-MEP antigen to detect anti-HIV antibodies further by testing it against BBI’s worldwide HIV performance panel (WWRB 302) consisting of 25 sera. Of these sera, 21 sera were HIV-1 positive, representing genotypes A, B, C, D, E, F, G, and O, from diverse geographical locations such as the United States, Spain, and several countries in Asia and Africa. Of the remaining four sera in this panel, two were HIV-2 positive and two were HIV negative (Table 3). Interestingly, the in-house immunoassay using r-HIV-MEP identified all 21 HIV-1 samples and the 2 HIV-2 samples. Further, the two sera that were HIV seronegative using five different commercial kits were also seronegative in the in-house assay. To examine specificity in the background of other infections, we evaluated the in-house immunoassay using BBI’s viral coinfection panel consisting of 25 sera (PCA 201). Of these, 9 were HIV seronegative while the rest (n = 16) were HIV seropositive, based on commercial assays. We tested 7 of the HIV-seronegative and all 16 of the HIV-seropositive samples using the r-HIV-MEP-based immunoassay. Many of these samples were also seropositive for hepatitis B virus (HBV), hepatitis C virus (HCV), and/or human T-cell leukemia virus (HTLV). The results are summarized in Table 4. Significantly, regardless of the presence or absence of antibodies to HBV, HTLV, or HCV, the results of the in-house immunoassay for HIV antibodies closely matched the results obtained with the commercial assays. The lone exception was provided by panel member 20. This serum, which scored as HIV positive with the commercial kits, was assigned as HIV negative using the r-HIV-MEP-based assay. A closer examination reveals that this discrepancy is attributable to this sample being a borderline specimen. The S/Co ratios, which must be ≥1.0 to designate a sample as seropositive, were 1.1 and 1.0 for the two commercial kits and 0.9 for the in-house assay.

Overall, the data show that the performance of our single r-HIV-MEP-based immunoassay is in near total agreement with the commercially available multiantigen-based anti-HIV ELA kits, namely, Abbott HIV-1, Abbott HIV-1/2, Genetic Systems HIV-1, Genetic Systems HIV-1/2, and Organon Teknika HIV-1.

**DISCUSSION**

Our earlier work has established the utility of recombinant MEps in the detection of infection by different pathogens (1, 2, 6). The present study is based on the premise that the use of a single diagnostic intermediate designed to have HIV-specific immunodominant epitopes from all known genotypes and expressed to high levels in an E. coli expression system could effectively address the issues of cost and specificity associated with the currently available multiple-antigen-based anti-HIV diagnostic assays. To develop this recombinant antigen, we focused on the HIV-1 and HIV-2 antigens shown in Table 1. The ability of these proteins to elicit humoral immune response has been well documented, and their antigenic determinants have been identified using a variety of different approaches (4, 13, 16). We selected immunodominant, linear, and phylogenetically conserved epitopes from these antigens for incorporation into the synthetic antigen r-HIV-MEP. In order for a synthetic MEP to be capable of efficiently recognizing HIV-specific antibodies, it is necessary that its constit-

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**TABLE 2. Evaluation of r-HIV-MEP-based indirect immunoassay using HIV seroconversion panel (PRB 931; Boston Biomedica Inc.)**

<table>
<thead>
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<th>S/Co value</th>
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<tr>
<td>09</td>
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<td>42</td>
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*Abbreviations: BBI = Boston Biomedica Inc.; MEP = minor envelope protein; S/Co = signal-to-cutoff ratio.*

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*a* Bleed dates for member IDs 01 to 05 were in August 1995; those for IDs 06 to 09 were in September 1995.

*b* Values indicate signal-to-cutoff ratios obtained using the in-house indirect immunoassay. The results using the in-house assay are indicated in parentheses. Samples with S/Co values <1.0 are considered positive.

*c* Values indicate signal-to-cutoff ratios provided by the panel supplier (Gen. Sys., Genetic Systems; OT, Organon Teknika) using the indicated commercial kits. S/Co values ≤1.0 are designated positive (+), and those with values ≥1.0 are designated negative (−).
Table 3. Evaluation of r-HIV-MEP-based indirect immunoassay using worldwide HIV performance panel (WWRB 302; Boston Biomedica Inc.)

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* a Gtyn, genotype.
* b Values indicate signal-to-cutoff (S/Co) ratios, provided by the panel supplier (Gen. Sys., Genetic Systems; OT, Organon Teknika) using the indicated commercial kits. S/Co values ≤1.0 are considered positive.
* c Values indicate signal-to-cutoff ratios obtained using the in-house indirect immunoassay. The results using the in-house assay are indicated in parentheses. Samples with S/Co values <1.0 are designated negative (−), and those with values ≤1.0 are designated positive (+).
* d Neg, negative.

Uorrent epitopes exhibit significant reactivity to HIV-infected patient sera worldwide. Since we wanted to use E. coli-based overexpression from the cost perspective, it was necessary to work with linear epitopes, so that when incorporated into the synthetic protein, they would presumably retain their immunoreactivity toward anti-HIV antibodies. Finally, the phylogenetically conserved epitopes would facilitate the recognition of multiple HIV types and groups. The selected epitopes, which ranged in length from 28 to 51 amino acid (aa) residues, were fused in frame using flexible tetracyclic linkers between adjacent epitopes. These linkers are preferred in designing flexible chimeric proteins (20). Computer modeling analysis of the MEP antigen which showed all the chosen epitopes to be fused in frame using flexible tetraglycyl linkers between adjacent epitopes. These linkers are preferred in designing flexible chimeric proteins (20).

We overexpressed the recombinant antigen in E. coli and purified it under denaturing conditions, as it was insoluble despite its fusion to thioredoxin. We then evaluated the efficacy of r-HIV-MEP as a diagnostic intermediate in an in-house Eu³⁺-9d-chelate-based indirect immunoassay. In this assay we used the recombinant protein as the capture antigen and panels of HIV-infected (commercially available) and normal human sera as test samples. We then used anti-human antibody labeled with Eu³⁺-9d-chelate as a tracer and monitored captured anti-HIV antibody through TRF. The results showed that our synthetic diagnostic intermediate could indeed recognize and bind to anti-HIV antibodies, elicited by both HIV-1 and HIV-2. However, it is to be noted that the design of r-HIV-MEP precludes differentiation of HIV-1 from HIV-2. As HIV exhibits a distinct geographical distribution, a worldwide HIV performance panel (WWRB 302) was used to evaluate if r-HIV-MEP could recognize the HIV-infected sera from different parts of the world. Panel members included specimens characterized as HIV-1 group M (subtypes A to G), HIV-1 group O, and HIV-2. Our results demonstrated that r-HIV-MEP was able to recognize antibodies to a diverse set of HIV infections from India and from other countries such as Argentina, China, Ghana, Ivory Coast, Mozambique, Spain, Thailand, Uganda, the United States, and Zimbabwe. The HIV seroconversion panel (PRB 931) was utilized to evaluate the sensitivity of the r-HIV-MEP in the immunoassay, and the results were found to be in complete agreement with those of the best-performing Abbott HIV-1/2 EIA kit in the early diagnosis of anti-HIV antibodies in human sera. Our immunoassay was able to pick up panel member 6 of the HIV seroconversion panel (PRB 931), which showed immunoreactivity with the Abbott HIV-1/2 EIA kit only and not with other EIA kits. The viral coinfection panel (PCA 201), on the other hand, was used for the evaluation of specificity of the r-HIV-MEP in the immunoassay. This single diagnostic intermediate performed as well as the other commercial anti-HIV kits from Abbott, Genetic Systems, and Organon Teknika except for...
panel member 20 of the viral coinfection panel, which happened to be a borderline sample. The information regarding the subtype of this member is unavailable. Overall, the data attest to the utility of our designer antigen in detecting HIV infection, from diverse geographical locations, with high specificity and sensitivity. It is also useful in monitoring seroconversion. However, these are preliminary data and need to be corroborated with a larger number of serum samples. The use of fluorescent Eu\(^{3+}\)-9d-chelate enables simplified, rapid, and universal test protocols to be constructed for a wide range of analytical applications because of the ease of antibody labeling with Eu\(^{3+}\)-9d-chelate and easy measurement of fluorescence directly from the dry wells without adding any substrate or stopping the reaction. Though the current assay takes a few hours, it has the potential to be adapted to a rapid test format.

In conclusion, the high density of HIV-specific, phylogenetically conserved, and immunodominant epitopes selected for designing the r-HIV-MEP contributed to a high degree of sensitivity and specificity. Further, our strategy of using a single recombinant MEP completely obviates multiple peptide synthesis and multiple protein expressions, and our Eu\(^{3+}\)-9d-chelate-labeled antibody as a tracer further simplifies the immunoassay. These factors, together with the high level of expression of r-HIV-MEP in E. coli and its single-step affinity purification design, make this approach highly cost-effective for anti-HIV screening in blood banks in most developing countries. The yield of purified r-HIV-MEP from 1 liter of induced culture is sufficient for \(\sim40,000\) assays.

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