

Evaluation of Different V3 Peptides in an Enzyme Immunoassay for Specific HIV Type 1 Group O Antibody Detection

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ABSTRACT

Strategies to discriminate group O from group M infections need to be improved. We have developed and evaluated an HIV-1 group O V3 peptide-based enzyme immunoassay (PEIA) for specific HIV-1 group O antibody detection among HIV-1-infected patients. Synthetic peptides, derived from the amino acid sequences of the V3 loop of 15 different group O strains and 7 group O consensus sequences, were evaluated in a PEIA against a panel of genetically confirmed group O ($n = 33$), group M ($n = 90$), and HIV-1 antibody-negative sera ($n = 17$). The best-performing PEIA(s) were then used to screen 134 sera of European and 336 sera of Cameroonian origin for the presence of anti-HIV-1 group O antibodies. The reactivity of reference ("gold standard") sera to individual peptides in the PEIA resulted in the selection of five different peptides with sensitivities (sens), specificities (spec), and test efficiencies (TEs) in the range of 90 to 100%. Improvement of the PEIA was obtained with simultaneous reactivity of at least two different peptides in separate wells of an ELISA plate, together with stringent criteria for positivity. We were able to select seven peptide combinations each with a sens, spec, and TE of 96.9, 100, and 99.2%, respectively. None of the 134 European and 4 (1.2%) of the 336 Cameroonian samples were group O positive in the optimized HIV-1 group O PEIA; this was confirmed by the repeated presence of reactives, in agreement with the present knowledge of group O infection distribution. Finally, we were able to develop a strategy with a higher TE (99.2%) than the previously used ANT-70 (98.5%) and ANT-70/MVP5180 (95.7%). Our results show that optimal specificity rather than optimal sensitivity makes the V3 PEIA a sufficiently accurate epidemiological tool to be useful in estimating specifically group O infection among HIV-1-infected patients.

INTRODUCTION

THE PHYLOGENETIC CLASSIFICATION OF HIV-1 into group M and group O is well established.¹ Geographically, group O infections have been documented mainly in Central Africa, al-

though group O infections have been reported from several other countries in Africa, Europe, and the United States.² These viruses present a public health challenge, as several commercially available serologic assays have failed to detect them.^{3,4} Furthermore, an HIV-1 subtype B Western blot misses group

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O infections in about 10% of specimens, thus hampering the confirmation of initially reactive HIV antibody results.⁵ Commercially available HIV antibody screening assays have been updated to correct the lack of sensitivity by the inclusion of group O immunodominant peptides. Still, we need to monitor the spread of HIV-1 group O viruses for seroprevalence estimation and further characterization. The latter might be useful in evaluating and possibly improving commercial HIV antibody detection kits.

Previous studies monitoring the prevalence of HIV-1 group O infections made use of an ANT-70 or ANT-70/MVP5180 V3 peptide-based enzyme immunoassay (PEIA), followed by confirmation with either a Western blot^{6,7} and/or INNO-LIA HIV-1 type O.^{8,9} These strategies are limited. On the one hand, the ANT-70 and MVP5180 V3 PEIAs yield some false-negative and false-positive HIV-1 group O antibody reactivity,⁸ leading to under- or overestimation of the real HIV-1 group O infection seroprevalence. On the other hand, the Western blot and INNO-LIA type O lack sensitivity and/or specificity as confirmatory assays. The value of reactivity with gp120 of ANT-70 on Western blot for confirmation of HIV-1 group O infection seems limited.⁸ In addition, the INNO-LIA HIV-1 type O results do not always fit with the true genetic character of the virus with which the patient is infected. It was demonstrated that the individuals whose sera react simultaneously with group O and group M peptides in the INNO-LIA HIV-1 type O are most often infected with group M virus.¹⁰

Strategies to discriminate group O from group M infections need to be improved. Taken into consideration the great rate of amino acid sequence divergence in the C2V3 region encoded by *env*, as well as the increase in the number of newly defined HIV-1 group O strains,² one can question whether the ANT-70 or ANT-70/MVP5180 V3 PEIA is still the most appropriate assay by which to screen specifically for HIV-1 group O infections among HIV-1-infected individuals.

Further studies on gold standard sera (i.e., sera from individuals proven to be infected with either group O or group M viruses, on the basis of sequence and phylogenetic analysis) are necessary to evaluate the intrinsic sensitivity and specificity of the HIV-1 group O PEIA (using V3 or other peptides) and HIV-1 group O serologic confirmatory tests.

In the present study we analyzed to what extent peptides mimicking V3 loop sequences of 15 different group O isolates, as well as 7 consensus peptides, or combinations of these peptides, might perform better in predicting group O infections in a group O V3 PEIA.

MATERIALS AND METHODS

Sera

Sera, obtained from 33 HIV-positive individuals whose infections were confirmed by sequencing and/or phylogenetic analysis to be HIV-1 group O, were tested in PEIAs using 22 different V3 loop peptides to evaluate their intrinsic sensitivity. The sera were collected at the same time the virus was isolated from peripheral blood mononuclear cells (PBMCs). These sera included the following (in parentheses is the genome fragment used to characterize the strains genetically): ANT-70 (full

genomic sequence) and VI 1755 (*env* V3)¹¹; CA9 and VI686 (*env* gp160, part of *pol*, and *gag* p24)^{11a,12}; 2901/94, 2902/94, and 320 GA (*env* gp41 immunodominant region) (L. Gürtler and M. Peeters, personal communications, 1997); and BCF01, BCF02, BCF03, BCF06, BCF07, BCF08, and BCF11 (*env* C2V3 and *gag* p24).¹³ The remaining 19 samples (AB193/HA, AB267/HA, AB341/HA, RUD, SBF04, 189GA, 320TCH, 1483/95, 4354/94, 5778/94, 6245/94, 6405/94, 6599/94, 8161/94, 8913/94, 2045, 2046, 2047, 2048) were all phylogenetically analyzed using the *env* C2V3 region (L. Gürtler and F. Simon, personal communications, 1997).

Ninety sera (genetically confirmed as HIV-1 group M) from infected Belgian individuals and 17 sera from HIV-1-seronegative Belgian blood donors (negative status determined by Ortho [Raritan, NJ] HIV-1/HIV-2 assay) were used to evaluate the intrinsic specificity of the HIV-1 group O V3 PEIA. Genotyping of the HIV-1 group M strains was done by heteroduplex mobility assay (HMA)¹⁴ and C2V3 sequencing. The subtype distribution was as follows: 25 A, 37 B, 9 C, 11 D, 5 F, 1 G, and 2 H.

The 33 group O- and 90 group M-infected sera, as well as the 17 anti-HIV antibody-negative sera, were used as gold standard sera.

Four hundred and seventy confirmed HIV-1 antibody positive sera collected in Europe and Cameroon were tested using the optimized HIV-1 group O V3 PEIA. The composition of the serum panel was as follows: 82 Cameroonian sera kindly provided by P. Ndumbe and screened with the Vironostika uniform II plus O kit (Organon Teknika, Durham, NC); 254 sera from HIV-1-infected Cameroonian blood donors, kindly provided by L. Zekeng and screened with ENZYGNOST HIV1/2, ENZYGNOST HIV-1, Western blot 1, and Western blot 2 assays (Behring, Marburg, Germany) and with an "in-house" Western blot 5180 produced as described by Gürtler *et al.*¹⁵; and 134 sera from the European AIDS Serosurveillance Program (EASP, Amsterdam, The Netherlands).¹⁶

Peptides

Twenty-two peptides consisting of 24 to 29 amino acid in the V3 region of HIV-1 group O (Table 1) were synthesized by Neosystem (Strasbourg, France) and used as antigens in HIV-1 group O V3 PEIAs. After synthesis, the peptides were >80% purified by high-performance liquid chromatography analysis. Peptides 1 to 15 are derived from the V3 loop sequences of the isolates indicated. A phenogram analysis was performed on 18 HIV-1 group O V3 amino acid sequences, each 25 amino acids long. Consensus sequences were designed on the basis of homologies among representatives of the clusters in the phenetic tree, as indicated in parentheses: EASP25a (CA9, VI686, MVP2901, BCF07, ANT-70); EASP25b (BCF02, PVP2902, MVP7851, VAU); EASP25c (BCF01, BCF11); EASP25d (MVP4354, MVP5180, BCF08); EASP25f (MVP2171, MVP8161); EASP25A (EASP25a, EASP25b, EASP25c, EASP25d); EASP25B (EASP25f, BCF03).¹⁷ The phenogram clusters the peptides according to amino acid similarity.

HIV-1 group O V3 PEIA

The HIV-1 group O V3 PEIA was performed as described previously.¹⁸ The criteria for the best group O V3 loop peptide(s) to be used in the PEIA are defined as the peptide(s) that

TABLE 1. TWENTY-TWO GROUP O V3 LOOP PEPTIDES WITH CORRESPONDING STRAINS AND AMINO ACID SEQUENCES

| Peptide | Strain | Sequence |
|---------|----------|---|
| 1 | MVP-5180 | C-E-V-Q-D-L-H-T-G-P-L-R-W-R-S-M-T-L-K-K-S-S-N-S-H-T-Q-P-R |
| 2 | MVP-8161 | C-E-I-Q-K-I-R-I-G-P-M-G-R-Y-S-T-Y-S-T-Y-A-K-N-S-G-G-S-A |
| 3 | MVP-2171 | C-K-I-Q-K-I-R-V-G-P-M-A-R-Y-S-T-Y-R-N-S-G-V-S-S |
| 4 | MVP-2901 | C-P-V-Q-E-I-R-I-G-P-M-A-W-Y-S-M-G-L-E-R-G-Y-T-N-K |
| 5 | MVP-4354 | C-E-V-Q-D-T-G-V-G-P-M-A-W-Y-S-A-G-L-K-R-D-K-N-S-T-S-R |
| 6 | CA9 | C-T-V-Q-E-I-R-I-G-P-L-A-W-Y-S-M-G-I-E-R-N-S-K-N-S |
| 7 | ANT-70 | C-D-I-Q-E-M-R-I-G-P-M-A-W-Y-S-M-G-I-G-G-T-A-G-N-S |
| 8 | BCF08 | C-T-I-Q-E-I-H-S-G-P-M-A-W-Y-S-L-G-L-K-R-N-T-T-V-R |
| 9 | BCF11 | C-K-V-Q-E-I-G-I-G-P-L-S-W-Y-S-M-S-I-A-E-D-S-A-K-N |
| 10 | BCF07 | C-K-V-Q-E-I-K-I-G-P-M-A-W-Y-S-M-G-I-E-N-E-N-I-P-D |
| 11 | BCF03 | C-G-I-K-Q-I-G-I-G-P-M-S-V-Y-S-G-S-L-A-D-L-G-G-N-N-N |
| 12 | BCF06 | C-K-I-Q-R-I-A-T-G-P-L-R-W-V-S-M-A-A-K-T-E-S-Q-N-T-G |
| 13 | PVP2902 | C-T-V-Q-E-I-K-I-G-P-M-A-W-Y-S-M-G-I-G-R-E-E-P-H-S-N-H |
| 14 | VAU | C-T-I-Q-K-I-M-A-G-P-M-A-W-Y-S M-A-L-S-N-T-K-G-D |
| 15 | VI686 | C-T-V-Q-E-M-K-I-G-P-M-A-W-Y-S-M-G-L-E-E-N-K-T-N |
| 16 | EASP25A | C-T-V-Q-E-I-R-I-G-P-M-A-W-Y-S-M-G-L-E-R-T-N-G-N |
| 17 | EASP25a | C-T-V-Q-E-I-R-I-G-P-M-A-W-Y-S-M-G-I-E-R-N-Y-T-N |
| 18 | EASP25b | C-T-V-Q-E-I-R-I-G-P-M-A-W-Y-S-M-G-L-S-R-T-N-G-S |
| 19 | EASP25B | C-K-I-Q-K-I-R-I-G-P-M-S-R-Y-S-T-Y-S-T-Y-L-V-S-S |
| 20 | EASP25c | C-S-V-Q-E-M-K-I-G-P-L-S-W-Y-S-M-S-L-A-E-N-S-S-K |
| 21 | EASP25d | C-E-V-Q-D-I-Y-V-G-P-M-A-W-Y-S-M-G-L-K-R-S-T-N-V |
| 22 | EASP25f | C-K-I-Q-K-I-R-V-G-P-M-G-R-Y-S-T-Y-S-T-Y-G-V-S-S |

score(s) the highest and lowest of group O V3 PEIA reactivities on sera from individuals proven to be infected with either genetically characterized HIV-1 group O and HIV-1 group M viruses, respectively. Sensitivity, specificity and test efficiency were evaluated for several combinations of two or three peptides, using different cutoff values (see Data Analysis) and positivity criteria.

Blocking HIV-1 group O V3 PEIA

To reduce cross-reactivity of HIV-1 group M antibody sera with the HIV-1 group O V3 peptides, the principle of block-

ing by excess peptide in liquid phase was applied, as previously reported.¹⁹ Two hundred microliters of diluted serum (1:100) was incubated with 10 μ l of an equimolar mixture of five different V3 loop group M peptides (subtype A, B, C, D, and E). The V3 loop group M peptides used were those described by Pau *et al.*²⁰ Two different peptide concentrations (0.5 and 0.75 μ g/ml) were used. As a control 5 μ l of serum was incubated with 10 μ l of blocking buffer. After incubation for 1 hr at 37°C the rest of the PEIA was performed as previously described.¹⁸ The percentage inhibition of binding induced by the five group M peptides for every serum sample tested was calculated as follows:

(OD without blocking – OD in presence of group M peptides)/OD without blocking

Confirmatory tests

INNO-LIA HIV-1 type O. All of the sera reactive by HIV-1 group O V3 PEIA were retested in a line immunoassay for the specific detection of antibodies to HIV-1 group O in human sera (INNO-LIA HIV-1 type O; Innogenetics, Zwij-

naarde, Gent, Belgium), in which biotinylated V3 peptides from different HIV-1 group O and M viruses (consensus HIV-1 group M, M-Mal; O-ANT-70; O-VI686; O-MVP5180) and biotinylated gp41 peptides (O-ANT-70; M-subtype B; M-subtype D) were applied as a streptavidin complex in parallel lines on nylon strips. The INNO-LIA HIV-1 type O assay was performed according to the instructions of the manufacturer. Positivity criteria were used as described previously.⁸

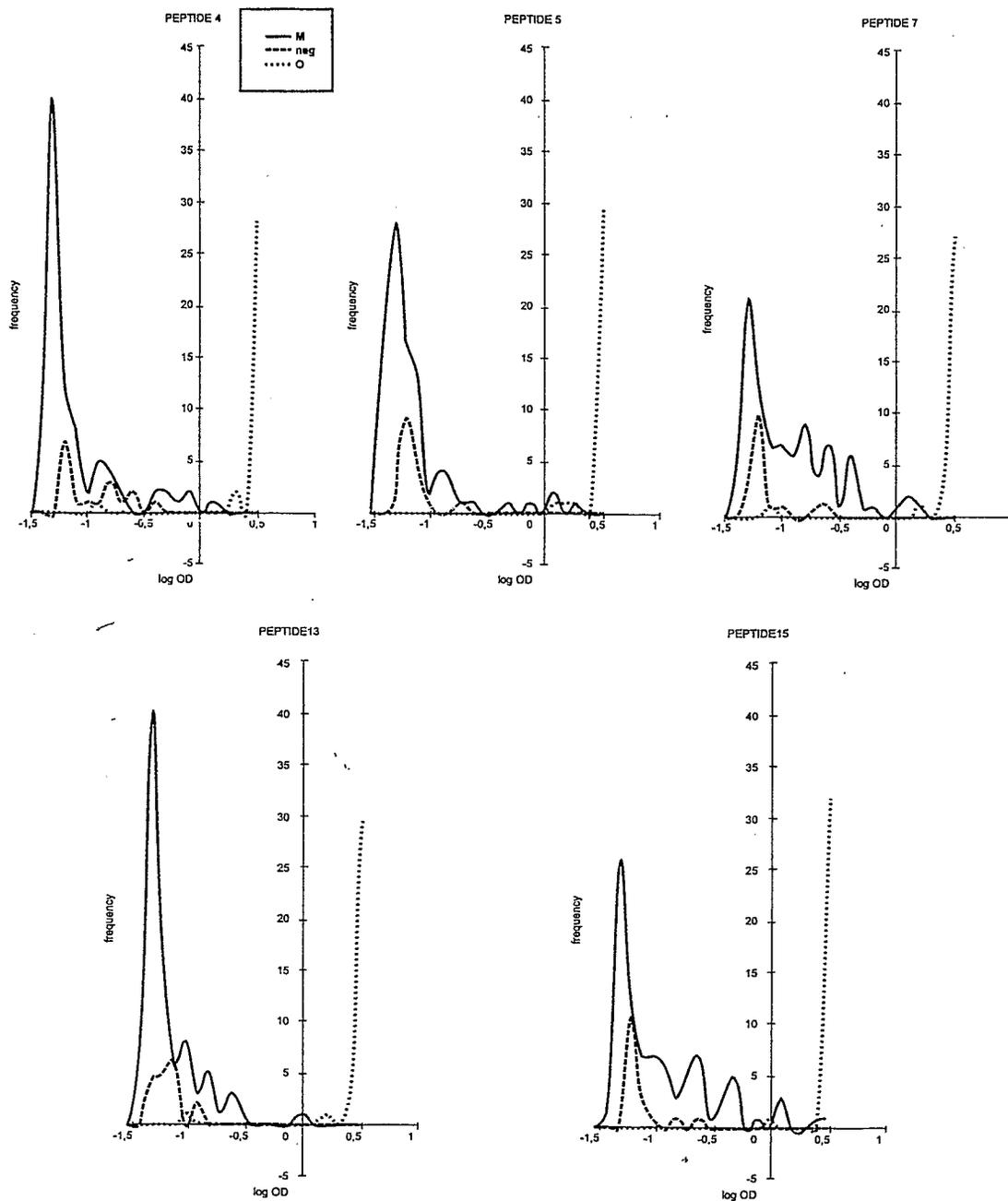


FIG. 1. Log OD distribution curves of the gold standard group O ($n = 33$), group M ($n = 90$), and HIV antibody-negative ($n = 17$) sera with the 5 best HIV-1 group O V3 PEIA.

Group O/group M-specific PCR

RNA was extracted from some sera reactive in the HIV-1 group O V3 PEIA, and reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out with specific primers to distinguish group O and M infection. Both group O-specific and group M-specific primers have been previously reported as achieving 100% sensitivity and specificity.^{10,21}

Differential diagnosis of HIV-1 group O and group M infection by PstI restriction analysis of the pol gene fragment

To gain more insight concerning the sensitivity and specificity of the confirmatory assays, *PstI* restriction analysis of the nested *pol* fragment (with newly designed *pol* primers) to distinguish HIV-1 group O from HIV-1 group M infection was performed as previously described¹⁰ on some HIV-1 isolates from individuals reactive in the HIV-1 group O V3 PEIA. *PstI* digestion of the group O PCR *pol* fragment (192 bp) resulted in 132- and 60-bp fragments, owing to a unique *PstI* restriction site (CTGCAG) present in group O and absent in group M. It allowed us to identify HIV-1 group O isolates with a sensitivity of 97.3% (36 of 37 tested) and a specificity of 100%, and group M isolates with a sensitivity and specificity of 100% (63 of 63 tested).¹⁰

Data analysis

Sensitivity, specificity, and test efficiency. Sensitivity, specificity, and test efficiency were calculated from the following formulas:

$$\text{Sensitivity (sens)} = \frac{\text{true positives}}{\text{true positives} + \text{false negatives}} \times 100$$

with true positives being the number of gold standard group O sera positive in the V3 PEIA, and false negatives being the number of gold standard group O sera negative in the V3 PEIA.

$$\text{Specificity (spec)} = \frac{\text{true negatives}}{\text{true negatives} + \text{false positives}} \times 100$$

with true negatives being the number of gold standard group M and HIV negative sera, negative in the V3 PEIA, and false positives being the number of gold standard group M sera plus HIV sera positive in the V3 PEIA.

$$\text{Test efficiency (TE)} = \frac{\text{true positives} + \text{true negatives}}{\text{true positives} + \text{false positives} + \text{true negatives} + \text{false negatives}}$$

Cutoff values. Cutoff (CO) values were determined from the percentiles of the OD distributions for each peptide:

COi:—The 95th percentile of the group M OD distributions (indicating 5% of the group M sera reacted with OD values higher than the COi)

COii:—The 5th percentile of the group O OD distributions (indicating 95% of gold standard group O sera reacted with OD values higher than the COii)

COiii:—The 10th percentile of the group O OD distributions (indicating 90% of gold standard group O sera reacted with OD values higher than the COiii)

Positivity criteria. Three different positivity criteria were used to evaluate combinations of two peptides: a sample is considered as putative group O antibody if only one peptide reacts (criterion A), both peptides react (criterion B), or one or two peptides react(s) (criterion C).

Six different positivity criteria were used to evaluate the combinations of three peptides: a sample is considered as putative group O antibody if only one peptide reacts (criterion D), two peptides react (criterion E), all three peptides react (criterion F), one or two peptide(s) react(s) (criterion G), two or three peptides react (criterion H), or one, two, or three peptide(s) react(s) (criterion I).

Statistics. The data were analyzed with the software Epi-Info 6 (Centers for Disease Control [CDC], Atlanta, GA).

RESULTS*Sensitivity, specificity, and test efficiency of group O V3 PEIA on gold standard sera of groups O and M as well as on sera of HIV-non-infected individuals*

Individual peptides. The 22 group O V3 loop peptides were evaluated in PEIAs for their capacity to bind antibodies in the sera of individuals proven to be infected with HIV-1 group O ($n = 33$), HIV-1 group M ($n = 90$), and HIV-1-negative sera ($n = 17$). The optimized HIV-1 group O V3 PEIA was then applied to sera from HIV-1-infected individuals from Europe and Cameroon.

We observed that the log OD distribution was not normal (Fig. 1), i.e., the means were different from the median values, and therefore, percentiles, rather than standard deviations, were used to describe the log OD distributions. The best peptides in terms of sensitivity, specificity, and test efficiency at each CO value were peptides 4, 5, 7, 13, and 15 (Table 2).

The highest sensitivity (100%) and specificity (100%) were obtained with peptide 5 (using COi) and peptide 4, 5, 7, 13, and 15 (using COiii), respectively. The highest test efficiency (98.5%) was obtained with COi and peptides 4, 7, 13, and 15 and with COii and peptide 13.

Combination of peptides. Reactivity patterns of a single gold standard serum to different peptides present in different wells of a microtiter plate were monitored. Combinations of two and three of the best individual peptides characterized above (4, 5, 7, 13, and 15) were examined. For each peptide combination, three cutoff values (COi, COii, and COiii) as well as all of the different positivity criteria were evaluated. Out of a total of 765 combinations (peptide I, cutoff, criteria) tested, 76 combinations were identified that allowed the monitoring of HIV-1 group O antibodies with 100% specificity, 96.9% sensitivity, and 99.2% test efficiency in genetically confirmed HIV-1 group O ($n = 33$), HIV-1 group M ($n = 90$), and HIV-1-negative individuals ($n = 17$). Compared with the HIV-1 group O V3 PEIA, using individual peptide reactivity results, the test efficiency and specificity were improved from 98.5 to 99.2% and from 96.2 to 100%, respectively. The highest test efficiency of 99.2% was obtained with pep4/pep13 and pep15/pep13 using criterion B with cutoff COii, as well as with pep7/pep13, pep5/pep7, pep5/pep15, pep15/pep13, and pep4/pep13 using criterion C with cutoff COiii (Table 2).

Blocking HIV-1 group O V3 PEIA

Attempts were made to reduce the level of cross-reaction, using methods described by Barin and colleagues.¹⁹ Antibody was diluted in an excess concentration of group M peptides prior to their incubation with solid-phase group O peptides in an attempt to compete out the cross-reactive antibodies. Six HIV-1 group O and 17 group O V3 PEIA cross-reacting HIV-1 group M sera (confirmed with INNO-LIA HIV-1 type O and PCR) were retested with the blocking HIV-1 group O V3 PEIA, using two different concentrations of group M peptides representing subtypes A, B, C, D, and E. In the first experiment, peptides 4, 13, and 15 were used in the blocking PEIA with group M peptides in the liquid phase at a concentration of 0.5 µg/ml. No inhibition greater than 50% was observed. The same experiment was done with group M peptides at a concentration of 0.75 µg/ml. A 50% inhibition was observed with three cross-reactive group M sera in a pep4/pep13 blocking PEIA and with one group O serum in a pep4 blocking PEIA. No percentage of inhibition higher than 37% was observed in the pep15 blocking PEIA (data not shown).

Use of the optimized HIV-1 group O V3 PEIA to screen HIV-infected individuals for HIV-1 group O infection

These seven peptide combinations with the use of the appropriate criteria and cutoff values resulted in higher test efficiencies (99.2% as compared with 98.5%) when reactivities to individual peptides were monitored. Consequently, these seven peptide combination strategies were used to screen Cameroon-

ian and European HIV-1 antibody-positive sera for the presence of HIV-1 group O antibodies. Among 336 HIV-1 antibody-positive sera from Cameroon, from 5 (1.5%) to 9 (2.7%) were reactive; among 134 EASP sera, from 1 (0.7%) to 7 (6%) were repeatedly reactive in the HIV-1 group O V3 PEIA (Table 3). Considering the HIV-1 group O PCR as the gold standard confirmatory test, the best percentage (60%) of confirmed repeated reactivities was obtained using combination 7/13 (COiii, C), followed by combination 13/15 (COii, B) (57%). Of a total of 470 HIV-1 antibody-positive sera tested, 21 sera (14 Cameroonian, 7 EASP) were repeatedly reactive with at least one of the seven peptide combinations in the HIV-1 group O V3 PEIA and were all tested by INNO-LIA HIV-1 type O. None of the EASP sera was confirmed as group O in the INNO-LIA HIV-1 type O. Only three of seven EASP samples were tested by RT-PCR and confirmed as group M infected (for four of them, there was not enough material available). Four Cameroonian sera were confirmed as group O, and one as a group O and M dual infection, in the INNO-LIA HIV-1 type O (Table 4). All of the Cameroonian samples were tested by RT-PCR. The four sera found to be group O positive in the INNO-LIA HIV-1 type O were confirmed as group O by RT-PCR (Table 4). The dually reactive sample was confirmed as an HIV-1 group M subtype A infection (data not shown). Of the nine group O-negative/group M-positive samples as determined by INNO-LIA HIV-1 type O, one was confirmed as a group M infection by RT-PCR; the *pol* gene of the others could not be amplified. Overall, the RT-PCR confirmed that HIV-1 group O antibody prevalence is 1.2% (4 of 336) in the Cameroonian sera, and 0% in the EASP sera.

TABLE 2. SENSITIVITY, SPECIFICITY, AND TEST EFFICIENCY OF THE BEST INDIVIDUAL AND SEVEN BEST GROUP O V3 PEPTIDE COMBINATIONS IN PEIA^a

| Peptides | COi ^b | Sens ^c (%) | Spec ^c (%) | TE ^c (%) | COii ^b | Sens ^c (%) | Spec ^c (%) | TE ^c (%) | COiii ^b | Sens ^c (%) | Spec ^c (%) | TE ^c (%) |
|------------------------|------------------|--------------------------|--------------------------|------------------------|-------------------|--------------------------|--------------------------|------------------------|--------------------|--------------------------|--------------------------|------------------------|
| 4 | 0.52 | 96.9 | 96.2 | 98.5 | 0.68 | 96.9 | 98.1 | 97.8 | 1.8 | 90.9 | 100 | 97.8 |
| 5 | 0.567 | 100 | 96.2 | 97.1 | 1.2 | 96.9 | 98.1 | 97.8 | 2.24 | 90.9 | 100 | 97.8 |
| 7 | 0.902 | 96.9 | 96.2 | 98.5 | 0.75 | 96.9 | 96.2 | 96.4 | 1.8 | 90.9 | 100 | 97.8 |
| 13 | 0.225 | 96.9 | 96.2 | 98.5 | 0.79 | 96.9 | 99 | 98.5 | 2.3 | 90.9 | 100 | 97.8 |
| 15 | 1.1 | 96.9 | 96.2 | 98.5 | 1.694 | 96.9 | 98.1 | 97.8 | 2.8 | 90.9 | 100 | 97.8 |
| 4/15 (C) ^d | 0.52/1.1 | 96.9 | 93.4 | 94.2 | 0.68/1.694 | 96.9 | 96.2 | 95 | 1.8/2.86 | 96.9 | 100 | 99.2 |
| 5/15 (C) | 0.567/1.1 | 100 | 94.3 | 95.7 | 1.2/1.694 | 100 | 97.1 | 97.8 | 2.24/2.86 | 96.9 | 100 | 99.2 |
| 13/15 (C) | 0.225/1.1 | 96.9 | 93.4 | 94.2 | 0.79/1.694 | 96.9 | 97.1 | 97.1 | 2.3/2.86 | 96.9 | 100 | 99.2 |
| 13/15 (B) ^d | 0.225/1.1 | 96.9 | 99 | 98.5 | 0.79/1.694 | 96.9 | 100 | 99.2 | 2.3/2.86 | 84.8 | 100 | 96.4 |
| 7/13 (C) | 0.902/0.225 | 96.9 | 96.2 | 96.4 | 0.75/0.79 | 96.9 | 96.2 | 95 | 1.8/2.3 | 96.9 | 100 | 99.2 |
| 7/5 (C) | 0.902/0.567 | 100 | 94.3 | 95.7 | 0.75/1.2 | 100 | 95.3 | 96.4 | 1.8/2.86 | 96.9 | 100 | 99.2 |
| 4/13 (B) | 0.52/0.225 | 96.9 | 100 | 99.2 | 0.68/0.79 | 96.9 | 100 | 99.2 | 1.8/2.3 | 87.8 | 100 | 77.9 |

^aWhen tested on gold standard group O ($n = 33$), group M ($n = 90$), and HIV antibody-negative sera ($n = 17$), using three different cutoff values.

^bCOi = 95th percentile of the group M sera OD distribution; COii = 5th percentile of the group O sera OD distribution; COiii = 10th percentile of the group O sera OD distribution.

^cSens (sensitivity) = true positives / (true positives + false negatives) × 100 (with true positives being the number of gold standard group O sera positive in the V3 PEIA, and false negatives being the number of gold standard group O sera negative in the V3 PEIA). Spec (specificity) = true negatives / (true negatives + false positives) × 100 (with true negatives being the number of gold standard group M sera + HIV sera negative in the V3 PEIA, and false positives being the number of gold standard group M sera + HIV sera positive in the V3 PEIA). Test efficiency (TE) = (true positives + true negatives) / (true positives + false positives + true negatives + false negatives).

^d(B) = a sample is considered as putative group O if both peptides of the combination react (C) = a sample is considered as putative group O if at least one peptide of the combination reacts.

The values in bold represent the best performances and the corresponding cut-off obtained with the peptide combinations.

TABLE 3. NUMBER OF REPEATED REACTIVE CAMEROONIAN AND EASP SERA TESTED POSITIVE BY INNO-LIA HIV-1 TYPE O AND RT-PCR^a

| Combination | Number of HIV-1 group O PEIA repeated reactives | | | Number of HIV-1 group O PEIA reactives tested positive in INNO-LIA HIV-1 type O | | Number of HIV-1 group O PEIA reactives tested positive in RT-PCR | |
|------------------|---|------|-------|---|-------|--|-------|
| | Cameroonian | EASP | Total | n | a (%) | n' | b (%) |
| 4/5 (COiii, C) | 8 | 0 | 8 | 5 (8) | 62.5 | 4 (8) | 50 |
| 5/15 (COiii, C) | 9 | 1 | 10 | 3 (10) | 30 | 3 (9) | 33 |
| 13/15 (COiii, C) | 6 | 1 | 7 | 3 (7) | 42.8 | 3 (6) | 50 |
| 13/15 (COii, B) | 7 | 0 | 7 | 4 (7) | 57 | 4 (7) | 57 |
| 7/13 (COiii, C) | 5 | 1 | 6 | 3 (6) | 50 | 3 (5) | 60 |
| 4/13 (COii, B) | 9 | 7 | 16 | 5 (16) | 29 | 4 (12) | 33.3 |
| 7/5 (COiii, C) | 7 | 1 | 8 | 3 (8) | 37.5 | 3 (7) | 42.8 |

^aScreening was done on 336 Cameroonian and 134 EASP serum sample. Twenty-one samples were reactive in at least one combination and were retested in the INNO-LIA HIV-1 type O; 17 samples group O positive by INNO-LIA HIV-1 type O were retested by RT-PCR. In four samples there was not enough material available.

Abbreviations: n, Number of HIV-1 group O PEIA repeated reactive sera tested by INNO-LIA HIV-1 type O; n', number of HIV-1 group O PEIA repeated reactive sera tested by RT-PCR; a, percentage of n HIV-1 group O V3 PEIA repeated reactives confirmed by INNO-LIA HIV-1 type O; b, percentage of n HIV-1 group O V3 PEIA repeated reactives confirmed by RT-PCR.

DISCUSSION

In this study 22 group O V3 peptides were analyzed by PEIA for their capacity to bind specifically to antibodies in the sera of individuals proven to be infected with genetically characterized HIV-1 group O, and not to bind HIV-1 group M antibodies. The highest test efficiency (ranging from 97.1 to 98.5%) using a single peptide was obtained with peptides 4, 5, 7, 13, and 15 irrespective of the cutoff values. Peptides 7 and 15, derived from the V3 amino acid sequences of HIV-1 ANT-70 and HIV-1 VI686, have previously been reported to be used successfully in other HIV-1 group O V3 PEIAs, as well as in a confirmatory INNO-LIA HIV-1 type O.⁸ Peptide 1, derived from HIV-1 MVP5180, scored a poor test efficiency (35–80%); however, V3 peptides derived from HIV-1 MVP5180 have previously been used frequently in V3 PEIAs as well as in confirmatory Western blots¹⁵ and the INNO-LIA HIV-1 type O.⁸ Our data suggest that V3 loop-derived peptides from HIV-1 MVP5180 not be included as antigens in PEIAs or confirmatory assays for monitoring specifically HIV-1 group O infections. None of the individual peptides allowed the detection of HIV-1 group O infection with 100% sensitivity and specificity.

To improve the test efficiency of the HIV-1 group O V3 PEIA, the simultaneous reactivity of a single gold standard serum with different HIV-1 group O V3 peptides present in different wells of a microtiter plate was monitored. Coating the same well with different peptides led to unpredictable variation in terms of peptide reactivity (data not shown).

Of 765 combinations tested, 7 (depicted in Tables 2–4) were selected for their higher performance compared with the individual peptides. Priority was given to those combinations for which the smallest number of peptides was involved, as well as to those with the highest cutoff values, to improve the specificity.

The seven optimal combinations, INNO-LIA HIV-1 type O, RT-PCR, and *Pst*I restriction site analysis, were used in a se-

quential way to analyze 336 HIV-1 antibody-positive Cameroonian and 134 EASP sera. Considering the repeated reactives as revealed by the HIV-1 group O V3 PEIA as true positives would result in an HIV-1 group O antibody prevalence of 1.5% (up to 2.7%) among the Cameroonian sera and of 0.7% (up to 6%) among the EASP sera. Considering the PCR group

TABLE 4. NUMBER OF INNO-LIA HIV-1 TYPE O GROUP O-REACTIVE SERA TESTED BY RT-PCR AND *Pst*I RESTRICTION SITE ANALYSIS

| Combination | Number of INNO-LIA HIV-1 type O reactives confirmed by PCR | | Number of INNO-LIA HIV-1 type O reactives confirmed by <i>Pst</i> I restriction site analysis | |
|------------------------------------|--|------------|---|------------|
| | n'' | c (%) | n''' | d (%) |
| 4/5 (COiii, C) | 4 (5) | 80 | 4 (5) | 80 |
| 5/15 (COiii, C) | 3 (3) | 100 | 3 (3) | 100 |
| 13/15 (COiii, C) | 3 (3) | 100 | 3 (3) | 100 |
| 13/15 (COii, B)^a | 4 (4) | 100 | 4 (4) | 100 |
| 7/13 (COiii, C) | 3 (3) | 100 | 3 (3) | 100 |
| 4/13 (COii, B) | 4 (5) | 80 | 4 (4) | 100 |
| 5/7 (COiii, C) | 3 (3) | 100 | 3 (3) | 100 |

^aThe values in bold represent the peptide combination giving the best performance.

Abbreviations: n'', Number of INNO-LIA HIV-1 type O, group O-reactive sera tested by RT-PCR; n''', number of INNO-LIA-O group O-reactive sera tested by *Pst*I restriction site analysis; c, percentage of n'' INNO-LIA HIV-1 type O, group O reactives confirmed by RT-PCR; d, percentage of n''' INNO-LIA HIV-1 type O, group O reactives confirmed by *Pst*I restriction site analysis.

O with a sensitivity and specificity of 100% as previously reported^{10,21} as the reference test, suggests that HIV-1 group O V3 PEIA using combination 13/15 (COii, B) can be considered the best choice. Combination 7/13 (COiii, C) yielded even 60% RT-PCR confirmed group O V3 PEIA results this combination missed one true positive sample. As such the confirmed prevalence rate of HIV-1 group O antibody positives among the Cameroonian sera tested is 1.2% (4 of 336) and 0% among the EASP sera.

The low group O seroprevalence in Cameroonian and EASP sera, as well as the cross-reactivity of HIV-1 group O V3 peptides with group M antibodies, may have contributed to the false positives. Blocking experiments with group M peptides in excess in liquid phase did not really improve the specificity of the V3 PEIA. The reduction in cross-reactivity of a few group M samples with group O peptides was accompanied by a significant decrease in group O sample OD values in blocking PEIAs. This may suggest that the majority of the cross-reactions are peptide specific and cannot be eliminated by this method. The role of other infections susceptible to cross-reactivity with HIV peptides is not excluded. For instance, *Mycobacterium tuberculosis*, herpes simplex, and varicella zoster have been described to share amino acid motifs with parts of the HIV envelope glycoprotein.²²

Theoretically, in a situation where 10,000 HIV-positive people, of which 1% are HIV-1 group O infected, are tested with our seven combinations (sensitivity of 96.9%, specificity of 100%), we expect to find a prevalence rate of 0.97 (3 false negatives, 0 false positives, with a total of 97 initial reactives to be expected). The predicted HIV-1 group O prevalence of 0.97% fits well with the real 1% prevalence. In the same situation, using ANT-70 alone (sensitivity of 90.9 specificity of 100%) as previously described or combination pep9/pep13 with an optimal sensitivity of 100% and a specificity of 99% (COiii, criterion C, data not shown) will give a less accurate prevalence rate of group O infections of 0.91 and 2%, respectively, instead of 1%. The HIV-1 group O antibody prevalence rates among HIV-positive sera documented so far were low and varied from 0 to 2%,⁸ which is in favor of our test strategy.

Only the HIV-1 group O PEIA repeated reactives were retested by INNO-LIA HIV-1 type O and group O/group M-specific RT-PCR and only the group O RT-PCR-positive samples were retested by *Pst*I restriction site analysis. The hierarchical testing format used in this study is not suitable for intertest comparison; however, we observed a discrepancy between the INNO-LIA HIV-1 type O and group O-specific RT-PCR results. Of 21 sera (14 Cameroonian, 7 EASP), repeatedly reactive with at least one of the seven combinations in the PEIA, 4 were considered HIV-1 group O antibody positive and 1 serum reacted with HIV-1 group O as well as group M peptides in the INNO-LIA HIV-1 type O. The first four were confirmed by PCR and *Pst*I restriction enzyme analysis. The latter was confirmed to be HIV-1 group M infected (subtype A), which supports our previous observations that some dually reactive sera (group M and O) as determined by INNO-LIA HIV-1 type O are not confirmed as type M and O dual infections.¹⁰ Peeters *et al.*⁸ confirmed that the majority of individuals whose sera react simultaneously with group O and group M V3 peptides in the INNO-LIA HIV-1 group O are infected only with an HIV-

1 group M virus as indicated by PCR.⁸ Serological dual group O/group M reactivity may be due to a broad immune response to a single serotype; to an aborted or suppressed group O infection in a group M-infected host; or to exposure or infection with a variant containing group O and group M epitopes. In addition, the sensitivity of the PCR *pol* primers can be questioned, considering the great variability existing within group O, and the relatively small number of group O viruses that have been analyzed to determine the sensitivity and specificity of these primers.^{10,21}

Exclusive studies of the sensitivity and specificity of the INNO-LIA HIV-1 type O, using sera of genetically proven HIV-1 group O-infected individuals, have not been performed thus far. However, in this study, 14 HIV-1 group O sera of our gold standard panel, 17 sera from this study, and 11 samples (described in Ref. 10) reactive in the group O V3 PEIA were tested in parallel with the INNO-LIA HIV-1 type O and by RT-PCR. The INNO-LIA HIV-1 type O confirmed 25 samples as group O infected (24 were typed as group O by RT-PCR; 1 could not be so typed, as its *pol* gene could not be amplified), 2 samples as group O/group M coinfections (1 was confirmed as group O; the second was confirmed as group M by PCR) and 15 sera as group M infected (the *pol* gene of 12 could not be amplified, but 3 were confirmed as group M infected by RT-PCR). These results show for the diagnosis of group O infections (on the basis of the 30 samples from which the *pol* gene could be amplified), a sensitivity and specificity of the INNO-LIA HIV-1 type O, compared with the group O/group M-specific RT-PCR, of 100% (95% confidence interval, 83.4 to 100%) and 75.0% (95% confidence interval, 21.9 to 98.7%), respectively. This is in accordance with the findings of others, who have shown that the majority of the INNO-LIA HIV-1 type O results so far are confirmed by RT-PCR.^{8,18} Using the INNO-LIA HIV-1 type O on group O V3 PEIA reactive samples improves the accuracy of group O infection detection. Besides, the INNO-LIA HIV-1 type O as a confirmatory test requires only serum and does not need an RNA extraction step as is required for PCR, which is logistically more demanding in terms of storage and reagents. For these reasons, this test remains a valuable tool for the diagnosis of an HIV-1 group O infection.

Moreover, the INNO-LIA HIV-1 type O scored the serum of a patient infected with HIV-1 BCF03 as HIV-1 group O positive. This serum remained negative by HIV-1 group O V3 PEIA, using our seven best combinations. One can be concerned that screening with two peptides in an ELISA is confirmed by other peptides on the INNO-LIA HIV-1 type O strip. However, the peptides coated on the strips are biotinylated and are possibly of different length than ours. This may influence the antibody binding in a positive way. In addition, the result of the INNO-LIA HIV-1 type O is always interpreted by comparing the sample reactivity with group O and group M antibodies, which confers a good specificity to the test.

CONCLUSION

To the best of our knowledge, this is the first time that reactivity of group O peptides against well-characterized group

M and group O samples was studied. We demonstrated that 7 peptide combinations (sensitivity of 96.9%, specificity of 100%, TE of 99.2%) performed better than any of the 22 individual peptides and the previous strategies using ANT-70 or ANT-70/MVP5180 in the V3 PEIA. Preliminary results from the screening of Cameroonian and European sera demonstrated that the combination of peptide 13 (PVP2902) and peptide 15 (VI686), followed by confirmation of repeated reactives, may be the best strategy to discriminate between group O and group M infections. Thus far the INNO-LIA HIV-1 type O has proven reliable in confirming a group O ELISA-positive result, but sensitivity and specificity analyses have not been performed yet on a sufficiently large panel of genetically confirmed group O and group M isolates. The PCR as a confirmatory assay is preferred but depends on the quantity and quality of nucleic acids in the sample and is logistically more difficult to implement. We suggest further specificity and sensitivity studies with larger panels of group M and group O sera from different geographic regions to improve the performance of the HIV-1 group O V3 PEIA, to monitor for HIV-1 group O infections among HIV-1-infected individuals.

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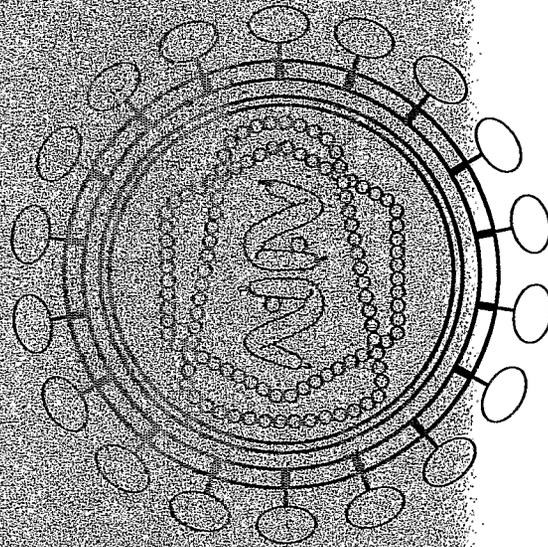
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