Serological Detection of Infection with Diverse Human and Simian Immunodeficiency Viruses Using Consensus env Peptides

SILVINA MASCIOTRA, DONNA L. RUDOLPH, GUIDO VAN DER GROEN, CHUNFU YANG, and RENU B. LAL

HIV Immunology and Diagnostics Branch, Division of AIDS, STD, and TB Laboratory Research, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333, and Division of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium

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Cross-species transmission has been shown to play an important role in the emergence of human retroviruses. We developed a generic enzyme immunoassay using synthetic peptides from gp41 and C2V3 consensus sequences (human immunodeficiency virus [HIV] type 1 [HIV-1] groups M, O, and N and the homologous region of simian immunodeficiency virus [SIV] strains from chimpanzees [SIVcpz], SIVcpzGABI and SIVcpzANT) to detect divergent HIV and SIV. A cocktail of peptides from gp41 and C2V3 (M-O) detected all HIV-1 group M and O sera and showed cross-reactivity with SIVcpz sera. Further, a mixture of C2V3 peptides (GABI-Ant) failed to detect HIV-1 infections but reacted with all SIVcpz sera, allowing discrimination of SIVcpz from HIV-1 infections. Since most SIVcpz sera cross-reacted with HIV-1 peptides, we next evaluated SIVcpz serum reactivity with rapid tests for HIV-1/2. SIVcpzANT and SIVcpzUS sera reacted with the Sero-strip and Multispot assays. Both tests are sensitive in detecting group M (97%, respectively), although Multispot has lower sensitivity for group O detection (67%) than does Sero-strip (100%). The limited volume and time required to perform these assays make them a generic tool for field screening. The env peptide-based assay and rapid tests should allow for the identification of emerging variants of HIV and SIV.

It is clear that zoonotic transmission plays an important role in the emergence of human retroviruses (4, 10, 11, 13, 14). Recent evidence has indicated that human immunodeficiency virus (HIV) type 1 (HIV-1) infection, the main cause of the worldwide AIDS pandemic (5), is the result of cross-species transmissions of simian immunodeficiency virus (SIV) into humans (4, 11). Based on analysis of isolates of SIV from chimpanzees (SIVcpz), the chimpanzee subspecies Pan troglodytes troglodytes has recently been identified as the natural host and reservoir for HIV-1 (4). These studies indicate that SIV strains from Pan troglodytes (SIVcpzGABI, SIVcpzGAB2, and SIVcpzUS) cluster closely with all HIV-1 strains, compared to SIVcpzANT, derived from Pan troglodytes schweinfurthii (4). Furthermore, while SIVcpz clusters closely with HIV-1 group N (4, 10), no simian counterpart has yet been identified for HIV-1 groups M and O. It appears that at least three independent cross-species transmissions gave rise to at least three known HIV-1 groups, M, O, and N (M for major, O for outlier, and N for non-M and non-O), and potentially additional cases may also exist. It is believed that although cross-species transmissions of nonhuman primate retroviruses to humans occur relatively frequently, the subsequent spread in the human population is a much rarer event (10, 14). Nevertheless, these zoonotic cases represent a unique opportunity to study the emergence of new human retroviruses in the human population and to study the genetic diversities of these viruses.

The adequate detection of new retroviruses emerging from cross-species transmissions may be hampered due to the lack of highly sensitive and generic tools that permit the identification of divergent HIV and SIV. In order to conduct systematic surveillance for the emergence of new HIV variants worldwide and to study zoonotic transmissions of HIV, highly sensitive and broadly reactive screening tools are urgently needed (7, 15). The ideal screening procedures require serologic tests that can detect divergent viruses, molecular detection-based assays for confirmation of infection, and an accurate method for classification into known HIV and SIV lineages, groups, and subtypes. We have recently developed generic molecular detection tools that permit the amplification of all known HIV-1 groups as well as all known SIVcpz strains, including the highly divergent SIVcpzANT (15, 16). Moreover, sequences from the amplicons allowed phylogenetic classification of HIV-1 and SIVcpz into expected lineages, groups, and subtypes (16). We now report a generic env peptide-based serologic assay that is reactive with a panel of sera representing infections with all known HIV-1 strains and the highly divergent SIVcpz strains. Further, selected commercially available rapid tests for HIV-1/2 are also cross-reactive with the highly divergent SIVcpz strains, thus providing a convenient serologic detection tool for rapid screening of HIV variants worldwide.

Synthetic peptides derived from the immunogenic C2V3 and gp41 regions (3, 6, 8), representing the consensus sequences for HIV-1 group M, group O, and group N and for SIVcpzGAB and SIVcpzANT, were synthesized by 9-fluorenylmethoxy carbonyl chemistry. The gp41 consensus peptides are as follows: group M peptide (amino acids 580 to 623, WGIKOLQARVLA VERYLKDOQQLLGWGCGSKLICTTATPVNSAW), group O peptide (WGIROLRARALLALETTITQQOPLLNLWGCGK KLYCITYSVKNVRTW), and group N peptide (WGIKOLQARVLKVLAVERYLRLDQQLLSLGCGSKTICYTVVPNWT); the SIVcpzGAB peptide is WGVKQLQARALLAVERYLQD QQLGILGWGCGSKAVCTTVTPWNNSW. The C2V3 consensus peptides are as follows: group M peptide (NTRKSVH IGPGQAFYATGDII), group O peptide (IDIOEKRIMIPMA WYSMIGGII), and group N peptide (NTRGGOVQIPAPMT FYNIEKII); the SIVcpzGABI peptide is NTRGEQVQIP GMTFYNIENVI, and the SIVcpzANT peptide is RTVRNLQI
GPGMTFYNYEIA T. These peptides were used in various combinations to develop a peptide-based assay.

Polyvinyl plates (Immulon II; Dynatech Laboratories Inc., Alexandria, Va.) were coated with 5 μg of synthetic peptide per ml (100 μl/well) in 0.01 M carbonate buffer (pH 9.6) and incubated overnight at 4°C. The plates were washed six times with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T). Excess reactive sites were blocked by the addition of bovine serum albumin (BSA)–PBS-T. Due to the high background reactivity previously seen with the peptides, 250 μl of 5% BSA–PBS-T was used per well for blocking. Following 2 h of incubation at room temperature, the plates were washed six times as described above. Serum samples were diluted 1:100 in 1% BSA–PBS-T, and 100 μl was added per well. The plates were incubated overnight at 4°C. Alternatively, the plates can also be incubated for 1 h at 37°C without compromising sensitivity. Following the incubation step, the plates were washed six more times to eliminate unbound antibodies.Fc-specific alkaline phosphatase-conjugated goat anti-human immunoglobulin G (Sigma, St. Louis, Mo.) was diluted 1:1,000 in 1% BSA–PBS-T, and 100 μl was added per well. The plates were incubated for 2 h at room temperature and washed six times, followed by the addition of 100 μl of 1 mg of p-nitrophenyl phosphate (Sigma) substrate per ml. After 30 min of incubation at room temperature in the dark, the plates were read at 405 nm. The cutoff values were calculated as the mean optical density (OD) plus 3 standard deviations of normal control sera in respective peptide assays.

Samples tested in the present study are part of various ongoing studies throughout the world and were selected based on their HIV-1-positive results in various enzyme immunoadassay (EIA) kits. Plasma specimens from 153 HIV-1 group M-infected individuals were selected from Argentina (19 subtype B, 29 subtype F), China (3 subtype B, 2 subtype B'), Egypt (1 subtype B), Ghana (7 subtype A, 7 subtype G), Ivory Coast (1 subtype A, 2 subtype G), Mozambique (5 subtype C), Republic of Congo (1 subtype G, 1 subtype H), South Africa (4 subtype C, 1 subtype B), Thailand (13 subtype B', 19 subtype E), Uganda (5 subtype A, 1 subtype C, 22 subtype D), and Zimbabwe (10 subtype C). All samples were subtype based on phylogenetic analysis of the C2V3 or gp41 region as described previously (15). Plasma samples from six group O specimens were obtained from Boston Biomedica Inc., Boston, Mass. In addition, plasma specimens from chimpanzees infected with SIVcpzUS (Marilyn) and SIVcpzANT (Noah and Niko) were kindly provided by L. Artur and G. van der Groen, respectively.

We first examined the reactivity of group M sera with the consensus group M peptides. All 130 group M sera (10 subtype A, 21 subtype B, 13 subtype B', 20 subtype C, 21 subtype D, 14 subtype E, 25 subtype F, and 6 subtype G) reacted with the consensus group M peptide, resulting in a test sensitivity of 100% (Table 1). The reactivity of group M sera was next examined with group O, group N, and SIVcpzGAB1 gp41 peptides. Although a high degree of cross-reactivity of group M sera with group N (92%) and SIVcpzGAB1 peptides (94%) was observed, these sera showed less cross-reactivity with group O peptides (84%). In all cases, a few specimens (6 to 16%) were missed by one of the peptides (Table 1).

Since the C2V3 region represents another highly immunogenic region of the HIV-1 envelope, we next examined the test containing a mixture of gp41 and C2V3 peptides. Various combinations of peptides were used in an EIA format initially to test the sensitivity of antibody detection (data not shown). Three cocktail assays containing combinations of peptides were used for further analysis and included gp41 and C2V3 from HIV-1 groups M and O, gp41 and C2V3 from HIV-1 group N and SIVcpzGAB1, and C2V3 from SIVcpzANT for serologic detection and C2V3 from SIVcpzGAB1 and SIVcpzANT to discriminate specimens with SIVcpz-like viruses (Table 2). Well-characterized HIV-1 group M (n = 38) and group O (n = 6) serum specimens and specimens from chimpanzees infected with SIVcpzUS (Marilyn) or SIVcpzANT (Noah and Niko) were tested for their reactivity in these peptide assays. All 38 group M (5 each of subtypes A and C to F, 6 each of subtypes B and G, and 1 subtype H) and 6 group O sera reacted in the consensus M-O (gp41-C2V3) peptide assay (Table 2); no group N sera were available. A high degree of cross-reactivity was observed in the HIV-1 group N and SIVcpz peptide assay with both group M (37 of 38) and group O (6 of 6) sera. More importantly, serum from a chimpanzee infected with SIVcpzZUS, closely related to HIV-1 group N (4), and sera from chimpanzees infected with SIVcpzANT (Noah and Niko), known to be highly divergent from HIV-1 (4), were also reactive in the HIV-1 group M-group O cocktail assay (Table 2). Despite several amino acid substitutions in gp41 (nine amino acids of the 44-mer region), sera from chimpanzees infected with highly divergent viruses had antibodies cross-reactive with the group M env consensus sequences.

While the presence of cross-reactive antibodies provides a good serologic screening tool, it does not permit the identification of specimens that may contain closely related SIVcpz-like viruses. We examined whether an assay based on the C2V3 region of SIVcpzGAB1 and SIVcpzANT would permit differentiation between HIV-1 and SIVcpz. Indeed, all 3 SIVcpz serum specimens (2 SIVcpzANT and 1 SIVcpzUS) had antibodies (OD > 1) and 1 of the 38 group M sera had antibodies cross-reactive with SIVcpz peptides (Table 2). Sequence analysis of the subtype A specimen that cross-reacted with SIVcpz peptides revealed it to be truly subtype A in both the C2V3 and the gp41 regions. Taken together, these data provide evidence that a generic assay using gp41 and C2V3 consensus peptides provides a highly sensitive screening tool likely to detect infection with SIVcpz-related viruses. Furthermore, discrimination should be possible using SIVcpz C2V3-specific peptide assays in order to screen for potential variants of HIV and SIV in the human population.

### Table 1. Seroreactivity of HIV-1 group M plasma samples with HIV-1 and SIV gp41 peptides

| Plasma subtype | No. of samples tested | No. of samples reacting with the following gp41 peptides: |
|---------------|-----------------------|----------------------------------------------------------|
|               | Group M | Group N | Group O | SIVcpz |
| A             | 10      | 10      | 9       | 9       | 9 |
| B             | 21      | 21      | 19      | 16      | 20 |
| C             | 13      | 13      | 12      | 12      | 11 |
| D             | 20      | 20      | 20      | 20      | 20 |
| E             | 21      | 21      | 19      | 11      | 20 |
| F             | 14      | 14      | 14      | 12      | 14 |
| G             | 25      | 25      | 20      | 23      | 22 |
|               | 6       | 6       | 6       | 6       | 6 |
| Total no.     | 130     | 130     | 119     | 109     | 122 |
| % Reactivity  | 100     | 92      | 84      | 94      | 94 |

* The subtype analysis was based on sequencing and phylogenetic analysis of the gp41 region as described previously (15). The samples included subtype A (6 Ghana and 4 Uganda), subtype B (19 Argentina, 1 Egypt, 1 South Africa), subtype B' (13 Thailand, 1 China), subtype C (10 Zimbabwe, 5 Mozambique, 4 South Africa, 1 Uganda), subtype D (21 Uganda), subtype E (14 Thailand), subtype F (25 Argentina), and subtype G (5 Ghana and 1 Ivory Coast).

**Seroreactivity of serum specimens with gp41 peptides representing consensus group M, group O, group N, or SIVcpz sequences.**
Seroreactivity of HIV-1 or SIVcpz plasma in synthetic peptide EIAs or rapid screening tests

| Subtype       | No. of specimens tested | Peptide-based assay       | Rapid test |
|--------------|-------------------------|---------------------------|------------|
|              |                         | Group M-group O (gp41 + C2V3) | Group N-SIVcpz (gp41 + C2V3) | SIVcpz (C2V3) | Sero-strip | Multispot |
| HIV-1        |                         |                           |            |             |           |           |
| Group M      |                         |                           |            |             |           |           |
| A            | 5                       | 5                         | 5          | 1<sup>d</sup> | 5         | 5         |
| B            | 6                       | 6                         | 5          | 0           | 6         | 6         |
| C            | 5                       | 5                         | 5          | 0           | 5         | 5         |
| D            | 5                       | 5                         | 5          | 0           | 4         | 4         |
| E            | 5                       | 5                         | 5          | 0           | 5         | 5         |
| F            | 5                       | 5                         | 5          | 0           | 5         | 5         |
| G            | 6                       | 6                         | 6          | 0           | 6         | 6         |
| H            | 1                       | 1                         | 1          | 0           | 1         | 1         |
| Group O      |                         |                           |            |             |           |           |
| US (Marilyn) | 6                       | 6                         | 6          | 0           | 6         | 4         |
| ANT (Noah)   | 1                       | 1                         | 1          | 1           | 1         | 1         |
| ANT (Niko)   | 1                       | 1                         | 1          | 1           | 1         | 1         |
| SIVcpz       |                         |                           |            |             |           |           |
| US (Marilyn) | 1                       | 1                         | 1          | 1           | 1         | 1         |
| ANT (Noah)   | 1                       | 1                         | 1          | 1           | 1         | 1         |
| ANT (Niko)   | 1                       | 1                         | 1          | 1           | 1         | 1         |

* gp41 of group M and group O plus C2V3 of group M and group O.
* gp41 of group N and SIVcpzGAB plus C2V3 of group N and of SIVcpzGAB1 and SIVcpzANT.
* C2V3 of SIVcpzGAB1 and SIVcpzANT. OD > 1.
* Sequencing and phylogenetic analysis of both C2V3 and gp41 regions revealed this specimen to be subtype A.

Since the search for HIV-1 variants is primarily focused in remote areas, specifically in central Africa, even simple peptide-based assays are difficult to perform on site. The ability of SIVcpz sera to react with consensus group M peptides, together with the ease of commercial rapid tests (most of which are based on the gp41 peptide), prompted us to examine the cross-reactivity of SIVcpz sera to two commonly used rapid tests with high sensitivity and specificity (Sero-strip HIV-1/2 [Saliva Diagnostic Systems, The Mendel, Singapore] and Multispot HIV-1/HIV-2 [Diagnostics Pasteur, Paris, France]). Serum specimens from a chimpanzee infected with SIVcpzUS (Marilyn), closely related to HIV-1 group N, as well as serum specimens from two chimpanzees infected with highly divergent SIVcpzANT (Noah and Niko) reacted in both rapid tests (Sero-strip and Multispot) (Table 2). While both assays were equally sensitive for the detection of SIVcpz-like viruses, the volume of serum required for the Sero-strip was much smaller than that required for the Multispot. As expected, both Sero-strip and Multispot had high sensitivities of detection for most HIV-1 group M sera; however, Multispot was less sensitive for group O serum detection than Sero-strip (Table 2). Previous studies with large numbers of HIV-1 group M and O sera have already established the sensitivities and specificities of these commercial tests (2, 12). Thus, it appears that most commercial tests (2, 12) have the capacity to detect antibodies against the wide HIV and SIV genetic spectrum. It remains to be seen if variants of HIV-1 that escape detection by these assays will emerge.

Thus, a simple algorithm for the screening and identification of HIV variants could include site testing of human and/or chimpanzee populations using rapid screening assays or a peptide cocktail assay comprised of HIV-1 env consensus sequences, followed by an SIVcpz C2V3-peptide based assay to define SIVcpz-like viruses. Once these candidate specimens have been identified, one could proceed with a detailed molecular analysis using generic primers for the highly conserved regions of the HIV-1 genome (9, 15, 16) and subsequent phylogenetic clustering analysis. We believe that this algorithm will provide a simple and effective approach for the identification of new and emerging variants of HIV-1 worldwide.

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