Reliable Detection of Individuals Seropositive for the Human Immunodeficiency Virus (HIV) by Competitive Immunoassays Using *Escherichia coli* - Expressed HIV Structural Proteins

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Reliable Detection of Individuals Seropositive for the Human Immunodeficiency Virus (HIV) by Competitive Immunoassays Using Escherichia coli-Expressed HIV Structural Proteins


We molecularly cloned the gag and env genes of the human immunodeficiency virus (HIV) and expressed fragments of these genes in Escherichia coli. Using the recombinant core and envelope proteins, we developed two competitive immunoassays (CIAs). Samples that recognized either the envelope or core proteins were considered positive for antibodies to HIV. This test system was comparable with western blot in detecting antibodies in patients with AIDS or AIDS-related complexes that were repeatedly reactive in the HIV screening test. All 360 individuals who were positive by western blot were positive by the CIA. A total of 844 samples repeatedly reactive by an ELISA screening test were negative both by western blot and by the CIA; 48 samples positive by ELISA, but negative or indeterminate by western blot, were positive by the CIA. Alternate research procedures verified the positivity of these individuals. These data indicate that the CIA described here may be useful as an adjunct or alternative to the western blot.

Although the blood screening tests for detecting antibodies to the human immunodeficiency virus (HIV; formerly known as HTLV-III or LAV) have been effective in identifying potentially infectious blood donors [1, 2], false-positive results do occur [3, 4]; it is therefore necessary to confirm the presence of antibody to HIV with a reliable, alternate immunologic test. The western blot is currently the test-of-choice as the alternate procedure. Western blot differs from the screening test in that it includes electrophoretically separated HIV antigens attached to a solid phase; thus virus-specific antibodies in the reactive serum can be identified with greater confidence. Like the screening tests, however, the western blot works by an antibody-capture principle, and false-positive results may occur with samples containing antibodies to contaminating, nonviral proteins or with samples containing immunoglobulins that bind nonspecifically to nitrocellulose [5-8]. Moreover, the western blot is subjective in nature. It is difficult to assess the specificity and sensitivity of the western blot in the same manner as a solid-phase ELISA in which a numerical value allows discrimination between reactive and nonreactive samples.

In this report, we demonstrate the efficacy of a quantifiable solid-phase competitive immunoassay (CIA) system (commercially available in Europe as Envacor®, Abbott Laboratories, North Chicago, III) that uses recombinant antigens and that can be used to confirm a positive result for HIV screening tests. This system consists of two separate immunoassays, one designed to detect antibodies to HIV gag gene products (core proteins) and the other to detect antibodies to HIV env gene products (envelope proteins). The antigens used in this immunoassay system were produced as recombinant DNA-derived proteins expressed in Escherichia coli. These assays provide independent determinations of the antibody response to the core and envelope proteins [9] and are highly specific and sensitive as compared with other established assays. This immunoassay system provides a testing procedure that could be used as a reliable alternative to the western blot for confirming antibody to HIV in sera.

Materials and Methods

Cloning and expression of HIV core and envelope proteins. HIV-infected HT 9 cells were obtained from the National Cancer Institute (Bethesda, Md.).
High-molecular-weight DNA was extracted from infected cells by using the standard procedure [10]. The DNA was partially digested by EcoRI and cloned into the EcoRI site of Charon 4A, a derivative of bacteriophage λ [11]. A recombinant library of 10^9 phages was screened with HIV DNA, and clones containing integrated proviral sequences were characterized and sequenced as described [12].

Segments encoding the gag gene included all of the amino acid residues of p24, as well as the COOH-terminal amino acid residues of p14 and the NH2-terminal amino acid residues of p31. Segments encoding the env gene included all of the amino acid residues of gp41 and the COOH-terminal amino acid residues of gp120. These segments were subcloned into the pUC 9 vector for expression of specific HIV proteins [13]. Expression of the cloned antigens was induced with isopropyl β-D-thiogalactopyranoside when the bacteria reached mid-log phase growth. After induction for 2 h, the bacteria were lysed by sonication as described [14].

CIA. Human IgG antibodies to HIV were purified by anion-exchange chromatography from human sera rich in antibodies to gp41 or p24, as described previously [15]. Polystyrene beads were coated with the HIV IgG, then exposed to a preparation of recombinant proteins containing either the envelope or core recombinant antigens. Additional IgG preparations from other seropositive individuals were conjugated with horseradish peroxidase (HRP) and then used in a modification of the procedures of Nakane et al. [16] and were diluted appropriately. The CIA for the envelope protein was initiated by adding test serum or plasma (50 μL) to the test beads (20 μL) in a 1:1 reaction well. 200 μL of diluted HRP-labeled human IgG antibodies to HIV envelope proteins were then added. A polystyrene bead coated with the recombinant HIV envelope protein was added. HRP-conjugated IgG and test samples were allowed to react for 1 h.

CIA for identifying HIV antibodies was performed by using the PicoTiterPlate system (Pentax®, Abbott Laboratories, Dallas). The amount of bound HRP-conjugated IgG was determined by adding per bead, 300 μL of a solution of 0.3% o-phenyleneediamine·2 HCl in 0.1 M citrate buffer (pH 5.5) with 0.02% H2O2. After 30 min of incubation at room temperature, the reaction was terminated by the addition of 1 N H2SO4. The color produced was inversely proportional to the amount of HIV antibody present in the test sample. The A405 was read on a spectrophotometer. A cutoff value was established as a function of both the positive and negative mean control values and was calculated as the sum of the control values divided by 2. The CIA for the core protein was performed in the same manner, except that HRP-labeled human IgG antibodies to the core protein and polystyrene beads coated with recombinant core protein were used. Positive and negative controls were included with each run.

Results

Population studies. Sera or plasma from various populations of subjects, including patients with AIDS, patients with ARC, blood donors seropositive for HIV, and unselected blood donors, were screened with the Abbott HTLV III ELISA (before its recent modification) and with the CIA test system for antibodies to HIV antigens (table 1). Western blot tests were performed on those samples that were repeatedly reactive in either the Abbott HTLV III ELISA or the CIA test system. Sixty-seven specimens from patients with AIDS were tested; all were positive by the CIA, whereas 66 (98.5%) of 67 were positive by the ELISA. Only 49 (73.1%) of the 67 were clearly positive by western blot when tested under our conditions, whereas 12 were indeterminate, and 6 were negative. Of the 186 donor specimens positive by screening for HIV that had been sent to the Abbott reference laboratory for confirmatory testing, 311 were positive both by western blot and by the CIA; 94 were positive only by the CIA, and 92 were negative. The remaining 31 specimens were positive by CIA but negative or indeterminate by western blot.

Table 1. Presence of HIV antibody, as determined by Abbott HTLV III ELISA, western blot, and CIA.

<table>
<thead>
<tr>
<th>Test</th>
<th>Subject group (no. of specimens tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patient groups with AIDS (67)</td>
</tr>
<tr>
<td></td>
<td>Blood donors (1186)</td>
</tr>
<tr>
<td></td>
<td>Unscreened blood donors (167)</td>
</tr>
<tr>
<td></td>
<td>Patients with other diseases (92)</td>
</tr>
<tr>
<td></td>
<td>Healthy controls (67)</td>
</tr>
</tbody>
</table>
| ELISA | 66                                     | 1186 | 2 | 0 | 0
| Western blot | 69 (92) | 311 (65) | 64 | 1 | 0
| CIA | 66 (98.5%) | 311 (65) | 64 | 1 | 0

NOTE. Data are given as no. of samples with positive results. Data for western blot are given as no. positive (no. indeterminate).

* We used results from the Abbott screening ELISA before it was modified in 1987 to improve its specificity and sensitivity; ELISA, enzyme immunoassay.

by the CIA. The two samples that were reactive by the screening test were negative by western blot and were considered to have had false-positive results by the screening test.

Evaluating discrepant samples. All of the specimens that were positive by western blot were positive by the CIA system (table 1). Those samples that were negative or indeterminate by western blot but positive by the CIA system were also positive by a CIA that used HRP-labeled mouse monoclonal antibo-
dies to gp41 or p24 instead of human IgG (data not shown). In addition, 25 donor samples that were negative or indeterminate by western blot and positive by the CIA were tested by the completed immunoprecipitation assay (RIPA) using purified, radiolabeled gp41 or p24. Fourteen of the 25 samples were positive by RIPA for antibodies to p24, whereas all were positive for antibodies to gp41 (data not shown).

Sensitivity studies on diluted samples. Serum samples from patients with AIDS; patients with ARC; and asymptomatic, healthy homosexual men sera were serially diluted by factors of two in normal serum. Each dilution was tested by the screening test, CIA, and western blot (table 2). The mean endpoint titer (greatest dilution yielding a positive result) of sera tested by CIA for the envelope protein was 2.5- to 28-fold higher than that obtained with the western blot. These titers were also 5.6- to 23-fold greater than titers obtained with the first-generation screening ELISA; the more recent, modified Abbott test has two- to fourfold greater sensitivity. The CIA for HIV core antibodies was less sensitive than that for the
Table 2. Endpoint titers for specimens, as determined by the ELISA screening test, CIA, and western blot.

<table>
<thead>
<tr>
<th>Subject group (no. of samples)</th>
<th>Screening test*</th>
<th>CIA using indicated protein</th>
<th>Western blot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Envelope</td>
<td>Core</td>
<td></td>
</tr>
<tr>
<td>Patients with AIDS (6)</td>
<td>88 (16-256)</td>
<td>930 (512-2048)</td>
<td>11 (0-64)</td>
</tr>
<tr>
<td>Patients with ARC (7)</td>
<td>296 (128-512)</td>
<td>8072 (4096-1600)</td>
<td>171 (0-112)</td>
</tr>
<tr>
<td>Asymptomatic persons (5)</td>
<td>296 (9-1024)</td>
<td>1664 (128-4096)</td>
<td>432 (0-2048)</td>
</tr>
</tbody>
</table>

NOTE: Data are given as mean (range). Reciprocal titer is defined as the inverse of the highest dilution at which any virus-specific band was detected.

Results were obtained with the first Abbott ELISA screening test.

antibodies to the envelope protein and was approximately equal in sensitivity to the screening test and western blot for detecting HIV antibodies in patients with ARC and in asymptomatic individuals. The CIA for the HIV core antibodies was, however, less effective than the other tests in detecting antibodies in patients with AIDS.

Distribution of CIA results. All sera from patients with AIDS and from blood donors positive for HIV by western blot were positive for antibodies to either envelope or core recombinant proteins by the CIA. Antibodies to the envelope recombinant protein were detected in >98% of individuals with various stages of HIV infection who were positive for antibody (table 3). In contrast, antibodies to core proteins were detected in 65%–72% of blood donors or patients with ARC, but were detected in only 21% of patients with AIDS.

Discussion

Because the screening tests for blood donors are designed for greater sensitivity than specificity, false-positive reactions would be expected. Presently, any unit of blood that is repeatedly reactive by the screening procedure is withdrawn from the donor pool and subjected to an alternate test to confirm the presence of antibodies to HIV. Donors are usually notified of their seropositivity only if the result of the alternate test is positive; this second test gives an added level of confidence in the accuracy of interpretation. The western blot has been the alternate immunosassay most commonly used to confirm the presence of HIV antibody in specimens that are repeatedly reactive by the screening test. The advantage of the western blot is that it separates viral proteins by molecular weight so that antibodies to specific HIV proteins can be identified, with particular attention given to antibodies to gp24 and gp41 [21]. Western blot can be as sensitive as the screening procedure, but the method requires a subjective interpretation. Moreover, the western blot uses viral preparations from the same source as those used in the screening test and it uses a test principle similar to that for the screening test; thus, false-positive results may recur during the confirmation test. In particular, the nonspecificity of antibody reactions at single bands on western blot is not uncommon [5-8]. Such reactions often occur at positions on gels comparable with those of viral proteins and have resulted in the need for more-restricted criteria in confirming an asymptomatic HIV infection. Consequently, most laboratories using the western blot as a confirmatory test require detecting reactions at more than one band on the blot for a result to be interpreted as positive.

Table 3. Distribution of antibodies to envelope and core proteins in specimens tested at Abbott Laboratories, by CIA.

<table>
<thead>
<tr>
<th>Subject group</th>
<th>Percent positive for antibodies to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Env and/or core</td>
</tr>
<tr>
<td>Seropositive blood donors (342)</td>
<td>100</td>
</tr>
<tr>
<td>Patients with ARC (47)</td>
<td>100</td>
</tr>
<tr>
<td>Patients with AIDS (67)</td>
<td>100</td>
</tr>
</tbody>
</table>

NOTE: Env, envelope protein; Core, core protein; +, positive; and –, negative. *Env* column represents env* and core* or core. **Core** column represents core* and env* or env.

Our first solid-phase confirmatory procedure consisted of a CIA system with whole-virus lysate being used as a target antigen [20]. Although this assay was successful, it did not provide specific information identifying which viral proteins were being recognized. We determined that the independent determination of antibodies to those to gp24 and gp41 would be needed as a criterion for a confirmatory procedure, and the sensitivity of one or both of these determinations should be equal to or greater than that of the screening test and comparable with that of the western blot. In addition, the procedures should be objective and standardized and should preferably use both an antigen source and a test principle that are different than those used in the screening test. The CIA using recombinant-derived antigens from the envelope and core proteins of HIV met these criteria. The high specificities of the CIAAs provided a level of confidence high enough that a duplicate positive reaction obtained by using one of the two CIAAs together with a positive reaction from a screening ELISA was defined as specific reaction to HIV.

The sensitivity of the screening tests for antibodies to HIV was first defined by the percentage of test results that were positive in a population of specimens from a population of patients with AIDS. In the present study, all 67 specimens from patients with AIDS were positive by the CIA, whereas one or more of these specimens was missed by the screening test or by western blot (table 1) when tested blindly in a routine diagnostic laboratory. A second indicator of sensitivity is the ability to detect all samples positive by western blot. In the present study, all 360 samples positive by western blot, including those from 311 donors and 49 patients with AIDS, were detected as positive by the CIA (table 1). In addition, 31 samples from donors and 17 samples from patients with AIDS that were positive by the screening test but were indeterminate (one band) or negative by western blot were positive by the CIA. The specificity of these reactions in the CIA was evaluated; results indicated that they were true positives.

Sensitivity has also been evaluated by determining the endpoint titers of known positive samples. Serial dilutions of specimens from asymptomatic donors should carry more weight than those from patients with AIDS in evaluating a test that will be used primarily in a blood bank setting. The CIA system produced endpoint titers greater than those for either the screening test or western blot for both specimens from patients with AIDS and from donors (table 2). After these data had been generated, Abbott modified its screening ELISA to improve specificity, and sensitivity was also increased by about two dilutions. Even so, the CIA procedure remains two-to-fourfold more sensitive than the screening test, and it is more sensitive, by several two-fold dilutions, than a western blot procedure that is routinely used in confirmation testing.

Specificity for the first screening procedures for HIV antibody was defined in terms of the percentage of nonreacting persons in a population of low-risk donors [20, 22]. By this definition, the specificity of the CIA system described here is 99.95% (table 1). Furthermore, false-positive results that might occur in the CIA may be of different biological or physical nature than those obtained with the screening test, because the two tests differ in both antigen source and test principle. Western blot has been the accepted standard of specificity. In this study, however, there were 48 specimens that were positive by the CIA but negative or indeterminate by western blot when they were tested without operator knowledge of other confirmatory results. Seventeen of these samples were from patients with AIDS and must be considered as true positives. The remaining 31 were donor specimens that had been positive in the screening test and were found to be positive by an alternate CIA that used monoclonal antibodies and recombinant antigens as reagents. Twenty-five of the 31 donor specimens were available for testing by RIPA [23, 24], and all were positive for antibodies to gp41, p24, or both. Thus the probability that the results for these 48 specimens are true positives is high. Therefore, the specificity of the CIA is established both for populations positive by the screening test and for those negative by the screening test. In additional studies performed in Europe, the CIA correctly identified all true-positive sera and all problematic samples in the group of negative sera when the sera were tested according to the manufacturer’s recommendations [23].

A potential disadvantage of the CIA system is that the recombinant proteins were derived from selected portions of the HIV genome and may not have detected all of the individuals who were truly positive for antibodies, as can be detected with the whole virus lysate. Our data indicate, however, that all true-positive samples, including those from patients with AIDS and those from blood donors whose blood was confirmed to be positive for HIV antibodies,
were equally positive when tested by methods using antigens from the purified virus or recombinant proteins. In other studies, successful tests for antibodies have been developed by using either recombinant proteins [26-29] or synthetic peptides [30] derived from the env gene or by using recombinant proteins derived from the gag gene [31].

In the present study and in European studies using this C1A [25, 32], 98% or more of the samples positive by C1A were detected with the envelope recombinant protein, regardless of the stage of infection (table 3). This finding is in agreement with other studies that have indicated that antibodies to the envelope proteins are the most common markers of HIV infection [33-37]. There is no clear explanation why a few specimens were negative for envelope antibodies but were positive for core antibodies. We have identified two specimens that gave false-negative reactions; these appeared to be caused by rheumatoid factor. We have subsequently modified the test reagents to correct for the phenomenon [36]. It is also possible that false-positive results could occur for specimens from subjects infected with HIV-2 [38], but this possibility cannot be confirmed presently.

By the C1A using the core antigens, ~66% of the 342 samples from blood donors confirmed positive for antibodies to HIV and 72% of the 43 patients withARC were reactive, yet only 21% of 67 samples from patients with AIDS were positive for antibodies to core proteins (table 3). Other studies using this C1A also indicate that the detectability of antibodies to core proteins declines in the later stages of infection with HIV-1.

This observation is verified by several other studies using various immunological systems [8, 23, 32, 44]. It has been apparent that the loss of antibody to core proteins is often associated with HIV-1 antiserum [10, 34,41] and with a worsening prognosis. Additional studies will be required to establish the value of the C1A and HIV-antigen tests.

References


