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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

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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 complex that were repeatably 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 repeatably 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, Ill) 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 _Eco_RI and cloned into the _Eco_RI site of Charon 4A, a derivative of bacteriophage λ [11]. A recombinant library of 10^6 phages was screened with HIV cDNA, 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 p18 and the NH$_2$-terminal amino acids of p15. 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) according to a modification of the procedure 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 specimen diluent (20 µL) in a 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 compete for binding to envelope protein overnight (16–22 h) at room temperature (~25 C). The beads were then washed three times with distilled water (5 mL/wash) by using a Pentawash® (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_-phenylenediamine–2 HCl in 0.1 _M_ citrate buffer (pH 5.5) with 0.02% H$_2$O$_2$. After 30 min of incubation at room temperature, the reaction was terminated by the addition of 1 _N_ H$_2$SO$_4$. The color produced was inversely proportional to the amount

of HIV antibody present in the test sample. The A_{492} 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. Positive samples containing antibodies to the HIV envelope or core recombinant proteins competed efficiently with the HRP–labeled antibodies for binding sites on the recombinant antigen bound to the solid phase. Samples having absorbance values equal to or less than the cutoff value were considered positive for antibodies to HIV. A sample reactive in either of the two tests (core- or envelope-protein CIA) was considered to be a positive verification of the presence of HIV antibodies.

Western blot. The western blot analysis was performed as described [17, 18]. Three standard controls were used in all western blot assays: a negative control (serum from a clinically normal, healthy individual), a positive control (serum from a patient with AIDS), and a diluted positive sample. The western blots were interpreted as positive if specific staining of viral proteins having molecular weights of 24 kilodaltons (kDa) and 41 kDa was observed. Alternatively, the blots with specific staining of either gp41 or p24 proteins in addition to proteins at 55 kDa and 65 kDa were also interpreted as positive. Samples with reactivity at 24 kDa or 41 kDa only were defined as indeterminate.

Mouse monoclonal CIA. A CIA system based on mouse monoclonal antibodies to HIV was developed to test specimens for which results by western blot and CIA did not agree. This system used the same components as the CIA described above, except that HRP–labeled mouse monoclonal antibodies to p24 or gp41 were substituted for the HRP–labeled human HIV antibodies. The monoclonal antibodies used were uniquely reactive with their corresponding native or recombinant protein targets [19]. Because of the high degree of specificity of the monoclonal antibodies, the monoclonal CIA could be expected to eliminate the false-positive reactions in the polyclonal CIA system.

Test specimens. Blood specimens from normal volunteer blood donors, patients with diseases unrelated to HIV infection, patients with AIDS, pa-

tients with AIDS-related complex (ARC), and blood donors seropositive for HIV were evaluated. The donor specimens (blood or plasma) positive for HIV were sent to Abbott Laboratories from various blood banks throughout the United States. This group consisted of 1186 specimens and were the repeatably reactive specimens in the HTLV-III screening test [20] obtained from ~200 000 blood donors across the United States. All samples were tested under code.

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 the CIA test system for antibodies to HIV antigens (table 1). Western blots were performed on those specimens that were repeatably 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 code, whereas 12 were indeterminate, and 6 were negative. Of the 1186 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; 844 were negative by both test systems. The remaining 31 specimens were positive by CIA but negative or indeterminate by western blot.

Two of 1667 specimens from unselected blood donors were repeatably reactive by the screening test. Neither of these two samples was positive by CIA or western blot, and they were considered to have had false-positive results. In addition, one of the 1667 samples was positive by CIA but negative by both the screening test and by western blot. This sample was also considered to have false-positive results. Western blots were run only on those samples that were positive by either the screening test or the CIA.

Specimens from 92 patients with diseases unrelated to HIV infection, including 9 patients with atopy, 20 patients with rheumatoid arthritis, 37 patients with leukemia, 20 patients with systemic lupus erythematosus, and 6 patients with infectious mononucleosis were evaluated; 90 of these were negative by the screening test, and all 92 were negative

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

Subject group (no. of specimens tested)	Test		
	Abbott HTLV III EIA*	Western blot	CIA
Patients with AIDS (67)	66	49 (12)	67
Blood donors (1186)	1186	311 (65)	342
Unscreened blood donors (1667)	2	0 (1)	1
Patients with other diseases (92)	2	0	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; EIA, 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 antibodies 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 in the CIA, were tested for antibodies by a radioimmunoprecipitation 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 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 EIA; 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.

Subject group (no. of samples)	Screening test*	CIA using indicated protein		Western blot
		Envelope	Core	
Patients with AIDS (6)	88 (16–256)	939 (512–2048)	11 (0–64)	117 (4–512)
Patients with ARC (3)	299 (128–512)	6827 (4096–8192)	171 (0–512)	234 (64–512)
Asymptomatic persons (5)	296 (8–1024)	1664 (128–4096)	422 (0–2048)	458 (16–2048)

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 repeatably 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 noti-

fied 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 immunoassay most commonly used to confirm the presence of HIV antibody in specimens that are repeatably 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 p24 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 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.

Subject group	Percent positive for antibodies to					
	Env and/or core	Env*	Core†	Env and core	Env⁺/core⁻	Core⁺/env⁻
Seropositive blood donors (342)	100	98	66	64	34	2
Patients with ARC (43)	100	100	72	72	28	0
Patients with AIDS (67)	100	100	21	21	79	0

NOTE. Env, envelope proteins; 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 such as those to p24 and gp41 would be needed among the criteria for a confirmation 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 combination of the two CIAs using recombinant-derived antigens from the envelope and core proteins of HIV met these criteria. The high specificities of the CIAs provided a level of confidence high enough that a duplicate positive reaction obtained by using one of the two CIAs 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 specimens 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 spec-

imens 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 twofold 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 with the CIA may be of a different biologic 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 [25].

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 (tables I and 2). 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 CIA [25, 32], 98% or more of the samples positive by CIA 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 consistent 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 subseqently 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 CIA using the core antigens, ~66% of the 342 samples from blood donors confirmed positive for antibodies to HIV and 72% of the 43 patients with ARC were reactive, yet only 21% of 67 samples from patients with AIDS were positive for antibodies to core antigen (table 3). Other studies using this CIA also indicate that the detectability of antibodies to core proteins declines in the later stages of infection [39–41]. This observation is verified by several studies using other immunoassay systems [18, 23, 42–44]. It has become apparent that the loss of antibody to core proteins is often associated with HIV p24 antigenemia [40, 41] and with a worsening prognosis. Additional studies will be required to establish the value of the CIA and HIV-antigen tests.

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