Early Detection of Antibodies Against rDNA-Produced HIV Proteins With a Flow Cytometric Assay

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There is evidence that some human immunodeficiency virus (HIV)-infected individuals have prolonged periods of seronegativity. A flow cytometric immunoreactive bead (IRB) assay is described for quantitative, simultaneous, and early detection of antibodies to HIV. Polystyrene beads of four diameters, each size coated with a different HIV recombinant DNA-produced protein (p24, p31, gp41, or gp120), bound anti-HIV antibodies detected with fluorescent antoglobulin. The IRB assay was performed on a panel of blood donor samples, many giving consistently false-positive enzyme immunoassay (EIA) and indeterminant Western blot (WB) results. The IRB assay proved as sensitive and more specific than currently licensed EIA and WB tests. Results on serial samples from eight HIV-infected individuals indicated that quantitation of anti-p24 by IRB assay may be useful in monitoring disease progression. Sequential pre- and post-EIA seroconversion sera from 35 HIV-infected homosexual men were tested by the IRB assay using IgM- and IgG-specific fluorescent probes. All 35 cases were IRB assay positive for at least one rDNA-p either before (17 of 35, 49%) or at the time of EIA positivity. Eleven cases (31%) initially had only IgM anti-HIV, primarily to gp41 (17%). In two individuals, the IgM response was detected at least 18 months before EIA seroconversion. The IRB assay is a widely applicable analytic procedure, potentially useful in pretransfusion anti-HIV screening of blood.

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referred to as p31 is from the putative endonuclease region of the polymerase gene and is a fusion product with human superoxide dismutase (SOD). The gp120 antigen corresponds to the entire amino acid sequence (a.a. 26 through 510) of HIV-SF2, gp120, envelope gene product. This S. cerevisiae recombinant gp120 is nonglycosylated and is denatured during purification. The gp41 is also a nonglycosylated protein, corresponding to a.a. 557 through 677 of the envelope gene product that was produced in Escherichia coli as a fusion protein with human SOD.

**Immunoreactive bead assay.** Polystyrene beads (diameter 5, 7, 10, and 15 μm ± 3% SD, Seradyn Diagnostics, Indianapolis) were washed twice with distilled water and once with either 0.05 mol/L, pH 9.2 borate buffer (10 and 15 μm) or Ca2+/Mg2+ free Dulbecco's phosphate-buffered saline (PBS) pH 7.4, (5 and 7 μm) and then counted (Model ZF, Coulter Electronics, Hialeah, FL). Nonaivalent coating with the rDNA-p was performed at the following concentration per 2 × 10^5 beads in the appropriate buffer: 5-μm beads, 5 μg p31/mL; 7-μm beads, 5 μg gp120/mL; 10-μm beads, 15 μg p24/mL; and 15-μm beads, 10 μg gp41/mL. Coating was performed at 37°C for three hours followed by 18 hours at 4°C. After coating, the beads were washed twice in 0.15 mol/L PBS, pH 7.4, and free binding sites blocked with purified human serum albumin (HSA) in PBS at 10 μg/mL/2 × 10^6 beads for one hour at 37°C. Beads were washed twice in PBS-0.1% Tween 20-0.03% bovine serum albumin (PBS-Tw-BSA), counted, and mixed in equal numbers. For performance of the IRB assay, 4.0 × 10^5 mixed bead preparation was added to a 1-mL polystyrene centrifuge tube, and spun at 7,000 g for five minutes, and the supernatant was removed.

The beads in each labeled reaction tube were resuspended in 0.1 mL serum diluted 1:40 in PBS-Tw-BSA, and incubated for one hour at 37°C. The beads were washed three times in PBS-Tw-BSA, and the beads were placed in 0.1 mL FITC-F(ab')2 goat anti-human IgG (heavy and light chain specific; Caltag, San Francisco) diluted 1:25 in PBS-Tw-BSA. In some experiments, immunoglobulin class-specific reagents were used [FITC-F(ab')2, goat anti-human IgG or anti-human IgM, Fc specific, Caltag]. After a one-hour 37°C incubation, the beads were washed twice in PBS-Tw-BSA and once in PBS-BSA-0.1% sodium azide. The IRB specimens were kept at 4°C until analyzed, always within a 48-hour period, without any change in results.

Twenty-two thousand stained, mixed beads were analyzed for each specimen by flow cytometric analysis at 300 to 500 beads per second (Fig 1) (FACSCAN, Becton Dickinson, Mountain View, CA). The median channel number of the fluorescence peak (MCF) was used as the assay measurement unit. Positive thresholds were empirically based on values obtained for normal controls, and were >6 SD above the mean for each rDNA-p when anti-IgG (heavy and light chain specific) was used. When Fc specific, anti-IgG or anti-IgM was used to label beads, a positive threshold of three times the mean MCF of simultaneously assayed normal controls was used.

**Standardization of the IRB assay.** Preliminary studies were performed to assess the linearity, specificity, and variability of the IRB assay. Linearity was determined by assaying two HIV seropositive sera diluted from 1:40 to 1:11,000 and plotting the MCF v reciprocal serum dilution. The specificity of the IRB assay was evaluated in neutralization experiments. Diluted anti-HIV positive or negative serum was incubated for one hour at 37°C with free rDNA-p, cytomegalovirus (CMV) antigen, rDNA-hepatitis B surface antigen (HBsAg, Recombivax HB, Merck, Sharp, and Dohme, West Point, PA), or buffer and then the IRB assay was performed as described above. Stability of the rDNA-p-coated beads was evaluated by preheating for three hours at 45°C before the IRB assay was performed on HIV seropositive control sera. The analytic sensitivity of the IRB assay was evaluated by assaying serial dilutions of anti-HIV positive sera by both IRB assay and HIV-1 EIA. The anti-HIV positive sera were diluted with normal sera before testing.

**Other HIV-1 immunoassays.** The HIV-1 EIA, WB, and antigen assays used in this study are available commercially and were performed according to manufacturer's directions. The EIA assay (LAV EIA, Genetic Systems, Seattle) uses HIV (lymphadenopathy-associated virus) grown in the CEM cell line for HIV antibody detection. The WB procedure (HIV Western Blot Kit, DuPont, Wilmington, DE) uses HIV (human T cell leukemia virus-IIIB) propagated in H9 cells for HIV antibody detection. The HIV antigen assay (HTLV-III antigen EIA, Abbott Laboratories, North Chicago) is a solid-phase sandwich-type EIA that detects predominantly core antigen (p24) with a sensitivity of 30 to 50 pg/mL.
Air-dried acetone-fixed slides with HTLV-III-infected H9 cells were used to detect HIV specific antibodies. Uninfected H9 cells and known anti-HIV positive and negative sera were used as controls.

Statistical analysis. The Mann-Whitney U (exact) test was used to compare quantitative anti-p24 levels between asymptomatic and AIDS/ARC HIV-infected individuals.

RESULTS

Standardization of the IRB assay. Linearity was evident on log-log plots of MCF vs reciprocal dilution of HIV seropositive sera. A mean correlation coefficient of -0.98 ± 0.02 SD and mean slope of 0.61 ± 0.02 SD was found when the data for all four beads on both specimens were combined. Specificity of the IRB assay was evident in that the homologous free rDNA-p reduced MCF <50% for the corresponding rDNA-p-coated bead. Table 1 shows results from a representative specificity experiment for anti-HIV positive sera. Sera from patients who had only IgM anti-HIV antibodies showed similar specificity. The between-run variability of the IRB assay was <10% of the MCF for HIV positive sera and <15% of the MCF for HIV negative sera for each type of rDNA-p-coated bead. Stable attachment of rDNA-p to beads was suggested when preheating mixed reagent beads at 45°C for three hours resulted in a reduction of <15% in MCF with HIV positive sera for each of the four HIV specificities. The analytic specificity of the IRB assay compared favorably with EIA. Positive IRB signals could be observed at one or two doubling dilutions past the point where EIA became negative in representative experiments.

Performance of the IRB assay on a serum panel. The results of the IRB assay with the blood bank serum panel are shown in Table 2. A sample was considered HIV positive by IRB if three or more of the different antigen-coated beads were above the respective positive threshold. For possible increase in IRB assay sensitivity in screening for anti-HIV antibodies, a broadly reactive, fluorescein-labeled, anti-human IgG with strong light chain reactivity was used. Specimen groups I and III were considered true negatives, and group II was considered true positive. Unexpected reactivity to gp120 and/or p24 rDNAp was observed in a single serum from group III and in four sera from group IV. Because the reactivity in each of the specimens did not meet the criteria for positivity (reactive with three or more rDNAp), it was interpreted as equivocal. Therefore, the specificity of the IRB assay was considered 100%. Since all true positive specimens (group II) were correctly identified, the sensitivity of the IRB assay was also 100%. Variability in anti-p24 reactivity was observed among the 24 specimens from HIV-infected individuals (group II), with 5 of 24 (21%) being nonreactive to p24 in the IRB assay. A single sample that was EIA+, WB-EQ, was reactive to all four HIV antigens. The results are not included in Table 2 because this individual was indeed HIV culture positive and subsequently became WB positive. Furthermore, the panel of 21 problem sera, containing autoantibodies or alloantibodies, were all negative by IRB assay (data not shown).

Quantitation of anti-p24 during disease progression. We next investigated the quantitative attributes of the IRB assay using three to five coded, sequential specimens from each of eight HIV-infected individuals. Heavy chain-specific anti-human IgG was used in the assay for possible decrease of variability due to immunoglobulin class or type changes over time. Four of the individuals remained asymptomatic during the ~2-year period in which the specimens were obtained (Fig 2A) and four were clinically ill, having or developing AIDS or AIDS-related complex (ARC), also termed HIV disease (Fig 2B). Most striking was the finding of low and decreasing levels of anti-p24 antibody noted in the AIDS group (mean MCF of 1,433 ± 147 SEM; p <.01), most clearly evident in Fig 2. One of the asymptomatic individuals (A*) showed a marked drop in anti-p24 level and CD4* (T helper) lymphocytes without development of symptoms, and his clinical status will be followed closely. One of the patients (B*) in the AIDS group was initially asymptomatic, progressed to ARC, and finally to AIDS. The anti-p24 level decreased throughout this period of disease progression. Antibody levels to the other rDNA-p tended to remain elevated in both study groups (data not shown).

IgM and IgG anti-HIV responses during seroconversion. The IRB assay was next used to investigate IgM and IgG responses to specific HIV antigens on coded, sequential serum samples from individuals pre- and post-HIV seroconversion, determined by HIV EIA and WB. The results shown in Fig 3 are representative of the various patterns of seroconversion seen. The IRB assay was reactive for at least one of the four rDNA-p in 17 of 35 (49%) sera before these sera were positive by the licensed EIA. In 11 of these individuals (31% of all subjects), only an IgM anti-HIV response was initially detected, with six (17% of all subjects) of the 11 having only IgM anti-gp41 initially detectable. In 29 of 35 (83%) individuals, IgM was noted with or without IgG at some point during the seroconversion. In none of the cases was the EIA or confirmatory test (WB or IFA) positive before IRB positivity, even with a requirement in the IRB

Table 1. Specific Neutralization of Anti-HIV Detected in the IRB Assay

<table>
<thead>
<tr>
<th>Antigen Added*</th>
<th>p31</th>
<th>gp120</th>
<th>p24</th>
<th>gp41</th>
</tr>
</thead>
<tbody>
<tr>
<td>p31</td>
<td>12</td>
<td>381</td>
<td>161</td>
<td>6</td>
</tr>
<tr>
<td>gp120</td>
<td>101</td>
<td>68</td>
<td>147</td>
<td>14</td>
</tr>
<tr>
<td>p24</td>
<td>106</td>
<td>344</td>
<td>6</td>
<td>94</td>
</tr>
<tr>
<td>gp41</td>
<td>96</td>
<td>341</td>
<td>182</td>
<td>12</td>
</tr>
</tbody>
</table>

* Significant difference between pre- and post-HIV seroconversion sera (P < .01).
Table 2. Performance of IRB Assay on a Serum Panel

<table>
<thead>
<tr>
<th>Specimen Group</th>
<th>p31 (≥ 100)*</th>
<th>gp120 (≥ 180)</th>
<th>p24 (≥ 100)</th>
<th>gp41 (≥ 150)</th>
<th>IRB Positive Specimen (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. EIA (-), WB (-)</td>
<td>0/11 (0%)</td>
<td>0/22 (0%) (21.6 ± 20.9)</td>
<td>0/22 (0%) (12.7 ± 8.3)</td>
<td>0/22 (0%) (33.3 ± 13.6)</td>
<td>0</td>
</tr>
<tr>
<td>II. EIA (+), WB (+)</td>
<td>11/11 (100%) (271 ± 64)</td>
<td>24/24 (100%) (433 ± 145)</td>
<td>19/24 (79%) (766 ± 690)</td>
<td>24/24 (100%) (2.187 ± 537)</td>
<td>100</td>
</tr>
<tr>
<td>III. EIA (+), WB (-)</td>
<td>0/12 (0%) (23 ± 24)</td>
<td>0/61 (0%) (57 ± 95)</td>
<td>1/61 (1.6%) (18 ± 30)</td>
<td>0/61 (0%) (43 ± 26)</td>
<td>0</td>
</tr>
<tr>
<td>IV. EIA (+), WB (EQ)</td>
<td>0/4 (0%) (9.4 ± 1.6)</td>
<td>3/18 (16.7%) (46 ± 24)</td>
<td>4/18 (22.2%) (39 ± 36)</td>
<td>0/18 (0%) (40 ± 18)</td>
<td>0</td>
</tr>
</tbody>
</table>

*Positive threshold: (MCF), greater than which is considered positive.
†Sera reactive with three or more rDNA-p.
‡Number of specimens reactive/total number: (% positive).
§MCF ± SD.
Fig 2. IRB quantitation of IgG anti-p24 levels correlates with disease progression. The IRB assay was performed using FITC-anti-human IgG, Fc-specific, antiseraum in the labeling step on sequential sera (A) from four asymptomatic HIV-infected individuals with a mean CD4" lymphocyte count of 566/µL; and (B) from four ARC/AIDS patients with a mean CD4" lymphocyte count of 1.17/µL. Quantitation was in terms of MCF. Patients discussed in the text are indicated (*). The asymptomatic individual (A*) had not only a decline in anti-p24 but also a progressive decline in CD4" lymphocytes (350, 302, 306, to 172/µL).

DISCUSSION

The IRB assay is a sensitive technique for simultaneous identification and quantitation of antibodies to multiple HIV antigens. It appears to be more accurate than the commercially licensed EIA and WB assays used, with dramatically reduced numbers of false-positive and indeterminant or equivocal findings. The few false-positive reactions in the IRB assay were restricted to gp120 and p24 rDNA-p. These specimens should be considered IRB equivocal, because antibodies to less than three rDNA-p were present. False-positive anti-HIV reactivities have been noted for these and other HIV proteins in assays using either partially purified whole virus or rDNA-p. Although these data are encouraging, the actual usefulness of the assay for screening large numbers of low-risk individuals, such as blood donors, remains to be proven in clinical trials.

The quantitative attributes of the IRB assay are suited for following anti-p24 levels in HIV-seropositive individuals. With a single serum dilution, the dynamic range (signal/noise ratio) of the assay is large (~1,000-fold) as compared with standard EIA procedures (~40-fold). Antibody quantitation by EIA, IFA, or WB techniques often require serum dilution. Numerous studies have recently correlated decreasing anti-p24 antibody levels to disease progression, and such levels may prove valuable in future therapeutic strategies. Further investigation of the utility of the IRB assay for quantifying anti-HIV antibody levels in HIV-infected individuals is planned.

The finding of IgM seroconversion to HIV proteins before IgG is expected. However, the finding of IgM up to 18 months before IgG by the IRB assay or positivity by a licensed EIA assay was unexpected. Other researchers have also provided evidence that in some cases HIV infection may be followed by relatively prolonged periods of EIA seronegativity. Allain et al showed that HIV antigen could appear before a detectable antibody response. However, in our study, the IRB assay showed IgM anti-gp41 a year before the single antigen-positive specimen found. The present study cannot exclude the possibility that other techniques, such as the polymerase chain reaction or sensitive culture techniques, might detect HIV infection as early as or earlier than the IgM IRB assay. However, the IRB assay did identify infected individuals before the serum HIV antigen or licensed antibody assay. Most HIV-infected blood donors currently missed by EIA are ultimately revealed to be men at risk of sexually transmitted HIV infection, such as those we studied.

The clinical utility of the IgM IRB assay in early diagnosis of HIV infection in members of high-risk groups, including infants born to HIV-positive mothers, remains to be determined through prospective studies.
Antigen neutralization studies of IgM anti-HIV reactivity in sera lacking IgG anti-HIV indicated specificity of the IRB assay in early detection of HIV seroconversion. However, fluctuations in IRB IgM reactivity were noted in 2 of 35 (5.7%) seroconverters (subjects 3 and 7, Fig 3). At this point in the seroconversion process, anti-HIV levels were low; the variation could be due to variability of the assay and/or actual variability in patient antibody levels. In support of the second possibility, one individual (subject 8, Fig 3) was weakly EIA positive at seroconversion, then was EIA negative six months later, before becoming strongly EIA positive at 12 months (data not shown). In addition, denaturation of IgM anti-HIV due to freezing and thawing of the specimens could not be ruled out as a cause of the apparent variability observed in the two patients.

A sequence of events appears to be indicated by the findings shown in Fig 3, together with those on the remainder of the seroconversion panel, and the quantitative data for each antigen (not shown). IgM appears to a single antigen (usually anti-gp41), then to multiple antigens, followed by its simultaneous appearance with IgG to the same antigens. There is a broadening of IgG responses to include all four specificities, along with weakening and finally loss of IgM response. However, in samples reactive for both IgM and IgG, the presence of IgM rheumatoid factor or anti-Gm allotypic antibody was not excluded.

This study demonstrates the importance of detecting gp41 specific antibodies in HIV serodiagnosis. All HIV-positive individuals studied were IRB reactive for anti-gp41, generally at high levels. In ten of 35 seroconverters, IgM anti-gp41 was either the earliest antibody detected (six cases), or was found with other IgM anti-HIV specificities. Gaines et al\(^4\) showed that the order of appearance of anti-HIV antibody specificities was assay dependent with the earliest reactivity detected by radioimmunoprecipitation to gp160 and by WB to p24. Allain et al\(^4\) showed that anti-gp41 appeared before anti-p24 in an rDNA-p EIA assay, as occurred in the present study. Other researchers have shown that false-positive reactivity to gp41 is unusual as compared with other HIV proteins, particularly p24.\(^1\) The improved sensitivity and specificity of newer assays, which use synthesized gp41 determinants is becoming increasingly apparent.\(^3\)

Use of microspheres for flow cytometric based immunoassays was described previously.\(^4\) This is the first demonstration of the utility of the IRB assay for simultaneous analysis of more than two specific antibody responses to an infectious agent (HIV). The procedure can be adapted to detect HIV antigen, HIV-specific immune complexes, and other blood-borne infectious agents. Automation of this procedure will permit its use in testing large numbers of samples.

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